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Heading for a Breakdown: Assessing Evolution Through the Hybridization of Two Sexual Systems

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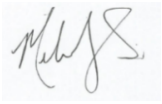

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Abstract

Hybridization is an important evolutionary pathway that has contributed to the world's vast biodiversity. In plants, especially angiosperms, hybridization is known to be an important mechanism for speciation, phenotypic divergence, and changes in reproductive systems. *Solanum* species present an ideal system to investigate how hybridization between two different sexual systems impacts the reproductive and phenotypic biology of the hybrid progeny. Hybrid seeds were acquired from crosses between Australian *Solanum* species *Solanum dioicum* (dioecious) and *S. ultraspinosum* (andromonoecious) in order to track what happens when you cross two plants with different sexual systems. Vegetative and floral morphological measurements were conducted, and the data was analyzed using an ANOVA and PCA to evaluate phenotypic differences across generations. Pollen tube growth was evaluated under a microscope using fluorescent microscopy technique to observe whether pollen tube growth occurred and whether it reached the ovary, providing insight into crossing success or failure. The only successful hybrids from the original crosses were those derived from *S. dioicum* as the pollen donor and *S. ultraspinosum* as the pollen recipient. Due to strong maternal effects, all F1 hybrids resembled *S. ultraspinosum*, thus all F1 plants were andromonoecious. The F2 and F3 hybrids demonstrate variability in inflorescence architecture, specifically the persistence of cosexual flowers in the staminate position of an andromonoecious inflorescence and the abortion of staminate buds, which may be suggestive of a change in sexual system. A principal component analysis supported that the F1 and F2 hybrids were

distinct from both parents, but were most similar to *S. ultraspinosum*, the pollen recipient, while the F3 hybrids clustered independently. In attempts to create an F3 and F4 hybrid generation, nearly all of our crosses have failed—suggesting that a hybrid breakdown is occurring. The observation of pollen germinating but failing to reach the ovary by fluorescent microscopy technique suggests that pollen tube abortion in the style is contributing to hybrid breakdown. This study should promote a better understanding of hybridization—a driving force in plant diversification—among Australian *Solanum,* a group in which hybridization is known to be widely possible but rarely confirmed in nature. Likewise, hybridization between taxa with two distinct sexual forms may shed light on the evolution of reproductive strategies in this clade.

Chapter 1: Introduction to the study system

Taxonomy and phylogenetic relationships of the study species

Solanum L. is the most species-rich genus in the economically- and ecologically-important Nightshade Family (Solanaceae) and one of the largest genera among all flowering plants (angiosperms), with ca. 1400 accepted species (Gagnon et al., 2022). While many of these species are concentrated in circum-Amazonian tropical South America, other areas of high species richness and many recent new species descriptions include Africa and Australia (Symon 1981; Särkinen 2013; Vorontsova et al., 2013; Gagnon et al., 2022).

In Australia, in particular, numerous new species have been described within the last 20 years from the sub-arid northern third of the continent known as the Australian Monsoon Tropics (e.g., Brennan et al., 2006; Martine et al., 2011; Bean, 2012; Martine et al., 2013; Barrett, 2013; Bean, 2016; Martine et al., 2016a; Martine et al. 2016b; Lacey et al., 2017; McDonnell et al., 2019; Williams et al., 2023). This area is home to a clade of ca. 45 currently-described species of "spiny *Solanums*" (i.e., *Solanum* subgenus Leptostemonum [Dunal] Bitter) belonging to the *S. dioicum* + S. *echinatum* Group sensu Martine et al. (2019).

Species of the *S. dioicum* + S. *echinatum* Group exhibit three types of sexual systems (see Symon, 1979; Anderson and Symon, 1989) addressed further below: a) cosexuality, in which every flower on every plant has both "male" (staminate, pollen producing) and "female" (pistillate/carpellate, egg/seed producing) parts (Figure 1A); b)

andromonoecy, in which each plant has flower clusters (inflorescences) consisting of a combination of male and cosexual flowers (Figure 1B); and c) dioecy, in which male and female flowers are segregated on separate male and females plants (Figure 1C) (Symon, 1970; Anderson and Symon, 1989).

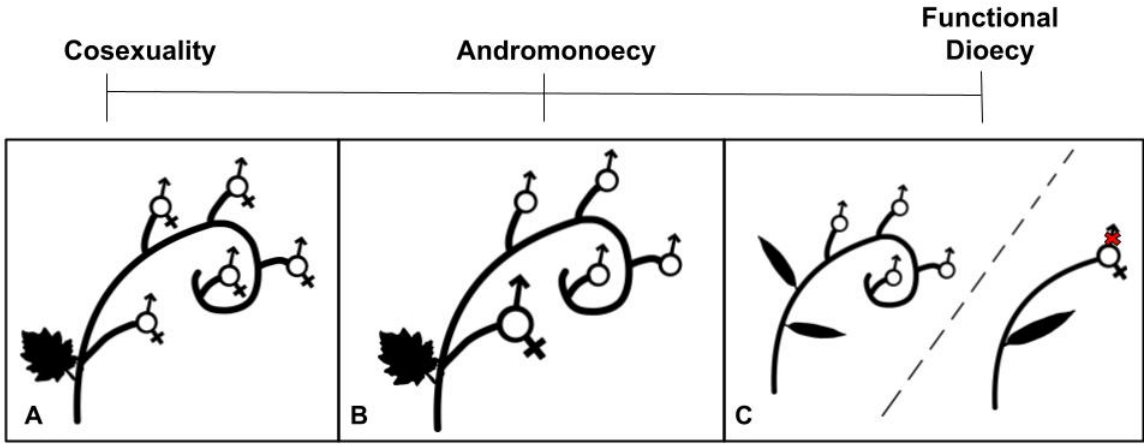

Figure 1 Depiction of sexual systems found in Australian *Solanum* species. Cosexuality (A), andromonoecy (sexual system of *S. ultraspinosum*) (B), and functional dioecy (sexual system of *S. dioicum*) (C) are represented. ⚥ represents cosexual flowers, ♂ represents staminate flowers, and ♀ represents carpellate flowers. The red X indicates loss of male functionality due to inaperturate pollen.

Recent assessments, including phylogenetic work (Bean, 2004; Martine et al., 2006; Martine et al., 2009; Martine et al., 2019; McDonnell and Martine, 2020), suggest that the dioecious and andromonoecious species within this group belong to three clades: 1) the "Kakadu dioecious clade" (two species [plus one forthcoming] of the upper Northern Territory); 2) the "Kimberley dioecious clade" (12 species occurring from the Kimberley Plateau of Western Australia to far northwestern Queensland); and the

"andromonoecious bush tomato clade" (14 species with roughly the same range as the "Kimberley dioecious clade").

This study focuses on hybrid progeny originating from crosses between two species with distinct sexual systems. *Solanum dioicum* W. Fitz. is a member of the "Kimberley dioecious clade" that is widespread (and variable) throughout a large distribution extending from western Northern Territory west to the northwestern coast of Western Australia. *Solanum ultraspinosum* A. R. Bean, of the "andromonoecious bush tomato clade," is narrowly endemic to a small region of Kakadu National Park (far northern Northern Territory) (Bean, 2016). These species express disparate sexual systems, belong to distinct clades, and do not overlap in range; yet they will hybridize when *ex situ* crosses are made (Hayes, 2018.)

Background on sexual systems evolution in *Solanum* and among angiosperms

In flowering plants, known as angiosperms, sexual systems are primarily characterized by the phenotype and functional role of flowers (Bawa & Beach, 1981). The sex of a flower is determined by the type of gamete it produces flowers containing only the egg cell are termed carpellate, while flowers containing only the sperm cell are termed staminate (Sundaresan & Alandete-Saez, 2010). Flowers that contain both the egg and sperm cell are known as cosexual (Ross, 1990). Sexual systems serve the evolutionarily favorable purpose of efficiently organizing each sexual role, largely resulting in the promotion of genetic diversity through genetic recombination, a process which promotes new

combinations of alleles (versions of genes that are characterized by having the same position on a chromosome) (Bawa & Beach, 1981).

Animal sexual systems are understood to separate the male and female sex, however, in plants such a separation is not the norm. In fact, the majority of flowering plants display cosexuality (Figure 1A), and thus are capable of self-pollination and self-fertilization (Barrett & Hough, 2013). Self-pollination occurs when pollen from the same flower or plant pollinates a recipient flower, and self-fertilization occurs when a plant fertilizes its own ovule (Wright et al., 2013). The capability of self-pollination (hereafter called selfing) presents an additional layer of complexity to angiosperm sexual systems due to genetic considerations. Selfing generally leads to lower reproductive success in most environments (i.e., is selected against) because it decreases genetic diversity, causing those genes to be inherited at a lower rate (Wright et al., 2008). Lower genetic diversity often results in an increase in deleterious mutations due to the higher rate of homozygosity (Wright et al., 2008). Because many harmful mutations only occur in the homozygous state (i.e., two of the same versions of an allele), the increase in homozygosity caused by selfing, a form of inbreeding, increases the prevalence of harmful mutations in a population (Johnston, 1998). However, in some cases, selfing may be advantageous or required for survival of species due to geographic isolation from other individual plants preventing outcrossing (cross pollination with genetically distinct individuals) (Herlihy & Eckert, 2002). This is a benefit known as reproductive assurance (Herlihy & Eckert, 2002).

Additionally, in flowering plants, the sperm cell is contained within the pollen grain (Raven et al., 2005). Pollen cannot be moved directly by plants, but rather requires vectors such as bees to move the pollen to recipient flowers (Lewis, 1955). These pollinators are attracted to the nutrients of floral rewards such as pollen or nectar (Irwin et al., 2004). Thus sexual system evolution is also influenced by the selective pressure of pollinators which may exert an influence on the size, color and shape of flowers, as well as the nutrient component of floral rewards (in the case of *Solanum* , pollen is the reward) by moving pollen between plants at differential rates dependent on the discernible quality of the floral reward, the flower's attractiveness, etc. (Fenster et al., 2004). Therefore, examining angiosperm sexual system evolution requires consideration of simultaneous selective forces.

Evolutionary selective forces promoting or impeding each sexual system

The most widely accepted explanation for the extensive presence of cosexuality in angiosperms is reproductive assurance, the ability of cosexual individuals to reproduce even when isolated from other individuals via selfing (e.g., Herlihy & Eckert, 2002). The fact that plants are sessile organisms compounds this effect (Lewis, 1955). However, a disadvantage of such a system is that the rate of selfing is potentially higher than outcrossing (the exchange of genetic material from separate individuals), which means plants may suffer the effects of inbreeding (Wright et al., 2008). One evolved response to offset self-pollination in plants is the evolution of self-incompatibility systems, which

prevent self-fertilization based on similar ancestry (Lewis, 1955; Haring et al., 1990; Newbigin et al., 1993; Pickup et al., 2019). Other mechanisms exist in angiosperms to prevent inbreeding, such as heterostyly in which the anther and stigma (sexual organs involved in pollen donation and receipt) are different lengths in order to prevent pollen of the same flower from reaching the stigma when released (Barrett, 1990), or dichogamy which involves the ripening of stamens and pistils at different times (Anderson and Stebbins, 1984). The sexual system known as dioecy presents an even more drastic response: the complete avoidance of selfing by segregating the sexes on separate individual plants (Pickup et al., 2019) (Figure 1C). This occurs by limiting pollen donation to individual male plants that are not capable of receiving pollen and limiting pollen reception to individual female plants who do not produce pollen capable of germinating (Anderson & Symon, 1989). Requiring cross pollination of two genetically distinct individuals is known as obligate outcrossing (Bawa & Beach, 1981). Indeed, the main advantage of dioecy may be the increase of genetic variation that results from the absence of selfing due to obligate outcrossing (Anderson & Stebbins, 1984.). Additionally, in environments with differential access to resources such as higher soil quality in some areas, it has been proposed that female individuals who require a higher energetic investment to grow and maintain seeds/fruit may be able to take advantage of more nutrient rich areas of soil (e.g. Finch et al., 2022).

In *Solanum* andromonoecy, in which every inflorescence typically produces a combination of a cluster of multiple staminate flowers subtended above a single cosexual flower at the base (Figure 1B), was proposed by Anderson and Symon (1989) as an

intermediate sexual system – meaning that it may serve as a pathway to other sexual systems like dioecy. Due to the large cluster of staminate flowers, andromonoecy presents the potential advantage of maintaining pollinator attraction and increased visits to cosexual flowers (Anderson & Symon, 1989). Additionally, andromonoecy in *Solanum* does display some sexual specialization by which reproductive resources can be conserved because each inflorescence contains only one cosexual flower, which requires greater energy to produce fruit (Anderson & Symon, 1989). In andromonoecious taxa (like one of the species examined in this study, *Solanum ultraspinosum*) self-pollination is certainly a possibility either by selfing within cosexual flowers or cosexual flowers receiving pollen from male flowers on the same plant. Thus, andromonoecious species may not experience the same genetic benefits that are assumed to manifest through obligate outcrossing in dioecious species (Anderson & Symon, 1989).

Dioecious populations can arise through multiple pathways (Barrett, 2013). Flower changes that might lead to dioecy are thought to be mediated by external factors such as pollen vectors visiting certain flowers more or less often, thus making them more functionally "male" or "female" (Beach, 1981). An additional mechanism by which dioecy can arise is through the loss of pollen functionality (Anderson, 1979; Anderson & Symon, 1989). Cosexual flowers may possess inaperturate pollen, which is considered nonfunctional due to the inability of the pollen to germinate (Anderson, 1979). Such flowers are said to be functionally female because they cannot fulfill the staminate role of sperm donation. While pollen production is energetically costly for functional female flowers, the ability to produce pollen is important because pollen is the exclusive floral

reward that these particular *Solanum* taxa provide to pollinators since they lack nectar (Anderson et al., 2023a; Anderson & Symon, 1989).

Prezygotic and postzygotic isolating mechanisms

Sexual systems can be impacted by prezygotic and postzygotic isolating mechanisms due to their role in preventing successful mating (Moyle, 2007). Prezygotic isolating mechanisms can occur before or after pollination in plants and prevent fertilization, while postzygotic isolating mechanisms occur after zygote formation (Moyle, 2007). Typically, prezygotic isolating mechanisms are present in sympatric species because of the fact that their similar geographic distribution causes mating to occur more often (Ortiz-Barrientos et al., 2009). Typically, two species will face lower reproductive success (a postzygotic isolating mechanism) as a result of mating, which, in this context, refers to delivery of pollen to the stigma. Multiple prezygotic and postzygotic isolating mechanisms exist in plants that prevent exchange of genes between different species (interspecific gene exchange), which are thought to be selected for mainly by the evolutionary pressure of energy conservation, since formation of inviable progeny is energetically costly (Dobzhansky, 1940; Howard & Harrison, 1993). Postzygotic barriers include hybrid sterility and hybrid inviability (discussed below) (Pickup et al., 2019). Of particular relevance to our study is pollen-pistil interactions, which comprise a post-mating but prezygotic barrier (Pickup et al., 2019).

Prezygotic barriers

Two types of widely studied pollen-pistil interactions are self-compatibility and self-incompatibility systems (Lewis, 1955). Self-compatibility is the proposed ancestral state to self-incompatibility (Lewis, 1955), and is present in *S. ultraspinosum*. Self-incompatibility results from interactions of similar alleles at the self-incompatibility (S) locus (Igic et al., 2006; Pickup et al., 2019). When alleles at the locus are identical, indicating similar ancestry, mating cannot occur (Igic et al., 2006). Self-compatible species lack the S allele; thus, mating is not prevented by similar ancestry (Anderson & Stebbins, 1984). It is hypothesized that sexual systems that lack self-incompatibility, such as dioecy, lead to higher crossing success due to the absence of allele interactions at the S locus, which might contribute to the benefit of dioecy (Anderson & Stebbins, 1984), although others have argued that dioecy is less efficient overall due to the fact that pollen movement must occur in the male to female direction making successful mating less likely (e.g. Karoly, 1994). Regardless, separation of sexes between individuals alters the dynamics of pollen-pistil interactions by making self-pollination impossible.

Postzygotic barriers

Hybrids are the result of cross fertilization between two different species (López-Caamal & Tovar-Sánchez, 2014). Broad definitions of hybrids include not only fertilization between different species, but also fertilization between genetically distinguishable populations (López-Caamal & Tovar-Sánchez, 2014). An important postzygotic barrier

preventing hybridization is hybrid seed inviability, which occurs when seeds produced from hybridization do not survive or successfully germinate (Coughlan, 2023). Hybrid seed inviability is an intrinsic reproductive barrier, meaning it is not affected by ecology, which could vary depending on environmental factors (Anderson et al., 2023b). An important consideration of hybrid seed development is the distinct ratio of parental contribution to the endosperm, a tissue involved in angiosperm development (Coughlan, 2023). The 2:1 ratio of maternal to paternal gene contribution to the endosperm is of particular relevance to hybridization because it likely means that reciprocal hybrid crosses also have reciprocal ratios of parental endosperm gene contribution.

Hybrid breakdown and hybrid vigor

The outcomes of hybridization have long been studied in plants, and were once thought to result only in intermediate phenotypes among hybrid progeny (Haartman, 1764; Roberts & Mendel, 1929). However, this is no longer thought to be true. It is now well established that hybrid phenotype is unpredictable (López-Caamal & Tovar-Sánchez, 2014). Two potential consequences of hybridization between species are hybrid vigor and hybrid breakdown. Hybrid vigor refers to improved growth in hybrid generations as compared to the parental generation (Chen, 2010). Hybrid vigor occurs as a result of an increase in heterozygosity (different alleles for a specific gene) following a hybridization event and can be observed as an increase in biomass of the hybrid progeny (Chen, 2010). Hybrid breakdown refers to reduced fitness in hybrids, especially manifesting in the F2

generation and beyond (Wissemann, 2007). While reduced fitness can be measured by multiple means, one way to measure it is reduced fruit or seed set in hybrid progeny (Wissemann, 2007). There are multiple mechanisms that could contribute to a hybrid breakdown, including shifting of pollen morphology and prezygotic reproductive barriers such as the inability of pollen to germinate or pollen tube abortion at the stigma or within the style (Levine & Anderson, 1986)

Chapter 2: Heading for a breakdown: Assessing evolution through the hybridization of two sexual systems

Introduction

Angiosperm sexual systems are among the most diverse of any organism (Barrett, 2010). While the function of sexual systems is relatively simple—to promote mating—the evolutionary struggles that are associated with promoting outcrossing and sufficiently attracting pollen vectors has created significant selective pressure to lead to this sexual diversification (Barrett, 2010). Cosexuality is the sexual system observed in most angiosperms, meaning every individual plant possesses cosexual flowers containing both the stamen and carpel within the same flower (Figure 1A); however, variation from this paradigm exists (Dufay et al., 2014). In addition to cosexuality, the *Solanum* genus, in particular Australian taxa, displays two other sexual systems of particular importance. The first sexual system is andromonoecy in which every inflorescence (arrangement of flowers on a plant) produces both cosexual and staminate flowers (Figure 1B); and dioecy in which staminate and carpellate flowers are found on separate plants (Figure 1C) (Anderson, 1979; Anderson & Symon, 1989). The presence of multiple forms of sexual systems in *Solanum* make it an ideal group within which to study the evolution of dioecy.

Sexual systems can evolve through multiple pathways, but one possible pathway is hybridization (Barrett, 2013; Barrett et al., 2010). Hybridization exists as a driving

force of plant speciation, which has broad impacts for the long-term viability of plant species (Soltis & Soltis, 2009). This impact is especially relevant in the context of climate change in which geographic shifts are increasing the frequency of species interactions, exacerbating hybridization rates (Chunco, 2014). Additionally, hybridization enables changes in multiple genes in one generation, whereas mutation, a major source of genetic variation, involves the change in one gene (Chunco, 2014). Because of the rapidity of climate change, similarly rapid phenotypic changes are required for species to persist (Chunco, 2014). Understanding the impact of hybridization on the sexual system of *Solanum* —an economically important genus—may help elucidate the mechanism by which separate sexes have evolved in plants. By analyzing the extent of hybridization between sexual systems and the range of potential barriers to reproduction, we may be able to understand the evolutionary forces that may promote or impede the evolution of dioecy and evaluate the role of andromonoecy in the transition to separate sexes.

The suggested evolutionary pathway from cosexuality to dioecy in Australian *Solanum* indicates that andromonoecy is an intermediate form of the two sexual systems (Martine & Anderson, 2007). It is proposed that the final transition to separate sexes functions as a means to increase genetic variation through obligate outcrossing, since all species in the *S. dioicum* + *S. echinatum* Group are genetically self-compatible and thus do not possess a mechanism by which to prevent selfing (Anderson & Symon, 1989). Hybridization is a known mechanism by which sexual system changes have occurred, and investigations of sub-dioecious populations of plants indicate that it may have arisen through hybridization of monoecious and dioecious individuals (Barrett, 2013; Barrett et

al., 2010). The purpose of this study is to evaluate what happens when you hybridize two species of Australian bush tomatoes with differing sexual systems, one andromonoecious (*Solanum ultraspinosum*) and one dioecious (*S. dioicum*). Ultimately, this enables an evaluation of whether hybridization functions as a means to shift from one sexual system to another, which has never been studied in Australian *Solanum.*

One of my major goals was to detect whether or not sexual system changes occurred as a result of the hybridization event. Two of the major distinctions between andromonoecy and dioecy, in Australian *Solanums*, are inflorescence architecture and pollen morphology (Anderson, 1979; Anderson & Symon, 1989). Therefore examining morphological characters of hybrid plants can provide insight into the pattern of inheritance across the generations and inform whether or not an intermediate phenotype developed, which might suggest sexual system changes (López-Caamal & Tovar-Sánchez, 2014). Pollen functionality may inform whether or not a shift to dioecy occurred, since inaperturate pollen is indicative of the state of functional dioecy in Australian *Solanums* (Anderson & Symon, 1989).

Methods

Ex situ hybrid plant crosses

Ex situ plant collection refers to the generation and growth of hybrids in a habitat other than their natural one, in this case being the greenhouse (Jaisankar et al., 2018). A major benefit of an ex-situ plant collection is that it enables hybridization of allopatric species,

which may not come into contact otherwise and thus have limited gene flow. However, a limitation of using an ex-situ plant collection is the inability to evaluate the success of the hybrids to survive and reproduce in a natural environment where selective pressure may alter the relative reproductive success of hybrids. For example, hybrid success is often increased in a highly disturbed environment (López-Caamal & Tovar-Sánchez, 2014). Thus, this study is not a question of hybrid success due to external or abiotic factors, but rather if hybrid offspring from parents with different sexual systems (andromonoecy and dioecy) are possible and what the resulting progeny might look like. Growing the hybrids in the greenhouse enabled consistent environmental factors, such as temperature, light condition, water level and soil nutrient composition.

First generation (F1) hybrid seeds of Australian *Solanum* were acquired from crosses conducted previously in the lab (Hayes, 2018) between two related species of "bush tomatoes" of the ~40-species "*S. dioicum* + *S. echinatum* Group" (as per Martine et al., 2019) endemic to the Australian Monsoon Tropics: *Solanum dioicum* (dioecious) and *Solanum ultraspinosum* (andromonoecious). Parental species were selected based on their high crossing success rate between sexual systems (Hayes, 2018). Subsequent crosses in each generation were generated by hand pollination. This occurred by removing anthers with forceps, tapping the forceps against a clean microscope slide, and making contact between the slide and the stigma of the recipient flower. Crosses were tagged with the date and pollen donor and recipient number and sex were noted. Crossing success was evaluated based on whether fruit was set. Fruit that did not produce germinable seeds were still considered successful cross attempts.

Germination percentage trail

The study species seeds were first treated with a Gibberellic Acid treatment to increase germination percentage (Deno, 1993). Seeds were soaked in 1000 parts per million gibberellic acid in the dark for twenty-four hours. Following the 24-hour treatment, the seeds were placed in petri dishes in the growth chamber between two sheets of filter paper in order to record germination rates and maintain moisture. The growth chamber mimics the arid conditions in Australia by first emitting 6 hours of light at a temperature of 20 ℃, followed by 14 hours of light at 32 ℃, finally followed by 4 hours of light at 30 ℃. The seeds were sprayed with water twice daily. Upon the emergence of the radicle, the seeds were transferred to a tray with damp soil and sprayed twice daily, while still remaining in the growth chamber. Upon growth, the seedlings were transplanted to the greenhouse in Bucknell's Rooke Biology building. Germination was observed for a period of 28 to 34 days; variation in this time period was due to planning difficulties during COVID. Germination percentage was calculated by dividing the number of seeds that displayed the emergence of the radicle in the specified time period by the total number of seeds planted.

Morphological measurements and analyses

Since this study was concluded in early spring, due to semester scheduling, many morphological traits for the F3 generation are absent due to lack of flower development at

time of conclusion. Nonetheless, statistical analyses were conducted with both parental, F1 and F2 generations, and included the F3 when possible.

Approximately five individuals per generation, including the parental, were measured for morphological characters with an emphasis on sexual characteristics (Table 1). Measurements of morphological characteristics were conducted using either a caliper, a tape measure, or using ImageJ (Schneider et al., 2012). The ImageJ protocol called for taking images of a leaf or dissected flower and setting the scale to a ruler in the image in order to measure a specimen's length, width, or area. All data was analyzed using the statistical software R (v. 4.2.1; R Core Team 2021) to generate boxplots, conduct 18 analysis of variance (ANOVA), and conduct a principal components analysis (PCA). Post-hoc Tukey analyses were conducted to determine which groups significantly varied and significance was determined at a p-value of 0.05. The Levene test and Shapiro-Wilk test were used to verify assumptions of the ANOVA (Levene, 1960; Shapiro & Wilk, 1965). Characters that were determined to not have equal variances by the Levene test, or determined to not be homogenous by the Shapiro test were transformed using the bestNormalize R package v. 1.9.0 (Peterson & Cavanaugh, 2020). Generations that were determined to significantly vary by the post-hoc Tukey analysis were indicated with letters A through D. The letter A was used to indicate *S. dioicum* and the letter B was used to indicate *S. ultraspinosum* when the two were significantly different from each other. Groups that share letters did not significantly vary. Sex differences were noted using colors for floral characteristics, but not vegetative.

Table 1 Morphological measurement types and methods of measurement.

Trait	Measurement Method
Vegetative characters:	
Plant height	tape measure
Stem prickle length	caliper
Stem diameter	caliper
Leaf length	ImageJ
Leaf width	ImageJ
Leaf area	ImageJ
Trichome counts	microscope
Floral characters:	
Corolla diameter	caliper
Calyx length	ImageJ
Calyx width	ImageJ
Petal length	ImageJ
Peduncle/Pedicel length	ImageJ
Anther length	ImageJ
Filament length	ImageJ
Stigma/Style length (cosexual/carpellate)	ImageJ
Ovary length (cosexual/carpellate)	ImageJ
Ovary diameter (cosexual/carpellate)	ImageJ

Pollen was collected by tapping anthers against a microscope slide and observed under a Nikon SMZ645 Stereomicroscope (Schneider et al., 2012) to determine whether it was porate or inaperturate. Flower presence and arrangement was assessed by counting the number of inflorescences and noting viable buds and flowers. Trichome counts were conducted by punching 0.5 cm area holes into both fresh leaves (F3 generation) and herbarium leaf samples (parental, F1 and F2 generations) and counting trichomes under a Nikon SMZ645 Stereomicroscope (Table 2).

Table 2 Trichome density count set up with the number of counts conducted and leaf disk source. Leaf disks were 0.5 cm.

Generation	**No. leaf disks** (Top and bottom)	**Leaf disk source**
S. dioicum (paternal)	3	Herbarium record
S. ultraspinosum (maternal)	5	Greenhouse
F1	2	Herbarium record
F2	2	Herbarium record
F3	5	Greenhouse
Total counts	34	

Fluorescence microscopy

To assess crossing success in the F2 and F3 generation, hand-pollinated crosses were examined after a period of 12 to 24 hours for pollen tube growth through the gynoecium using fluorescence microscopy. The technique was taught at the University of

Connecticut by Dr. Greg Anderson, a leading expert in plant reproductive biology who has done this work in *Solanum* for decades (Anderson, 1979; Anderson et al., 2023a; Anderson & Symon, 1989; Levine & Anderson, 1986). Hand pollinated flowers were collected after a period of 12 to 24 hours after initial pollination, then the gynoecium was removed and stored in 70% ethyl alcohol (EtOH). At least 24 hours after exposure to EtOH, gynoecium were rinsed in 30% ethyl alcohol to prevent cell lysis, then rinsed in water, and soaked in 0.8N NaOH for 24 hours. After this, crosses were transferred to tap water for a minimum of one day, then soaked in aniline blue (a stain that fluoresces callose tissue), mounted with glycerin and evaluated under a Nikon Labophot Fluorescence microscope.

Pollen tube growth was assessed at three levels of the gynoecium: at the stigma, within the style, and at the ovary. Evaluation under the microscope required distinguishing between phloem tissue and pollen tubes, which both fluoresce due to their callose composition. Identification of pollen tubes is made by looking for the presence of callose plugs. Images of the crosses were captured using a mounted Nikon Coolpix P6000 camera provided by Dr. Joe Moore in the Bucknell Biology Department.

Results

Ex situ hybrid plant cross germination percentage

The germination percentage is defined as the percentage of seeds that germinated during the germination cycle period. The germination percentage of each parent species was

94% for *S. ultraspinosum* with 47 of 50 seeds germinating, and 38% for *S. dioicum* with 19 of 50 seeds germinating in a 28 day germination cycle (Table 3). The F1 seeds with *S. ultraspinosum* as the pollen recipient species had a germination percentage of 92.5% with 37 of 40 seeds germinating, while the F1 seeds with *S. dioicum* as the pollen recipient species had a germination percentage of 0% with 0 of 50 seeds germinating in a 28 day germination cycle (Table 3). The F2 hybrids had a germination percentage of 84.7% with 548 of 647 seeds germinating in a 34-day germination cycle (Table 3). Finally, the F3 hybrids had a germination percentage of 36% with 27 of 75 seeds germinating in a 28-day germination cycle (Table 3).

Table 3 Seed germination percentage for all generations. Germination percentage is defined as the number of seeds that germinated during a set cycle divided by the total number of seeds prepared. Ult indicates *S. ultraspinosum*, while Dio indicates *S. dioicum*. Ult x Dio indicates *S. ultraspinosum* as the pollen recipient, while Dio x Ult indicates *S. dioicum* as the pollen recipient.

	Parental		F1		F2	F3
	Ult	Dio	Ult x Dio	Dio x Ult	Ult x Dio	Ult x Dio
Germination rate (%)	94%$^{+}$	38%$^{+}$	92.5%$^{+}$	0%$^{+}$	84.7%*	36%$^{+}$
Total number of seeds (n)	50	50	40	50	647	75

$^{+}$ = 28-day germination cycle
* = 34-day germination cycle

Ex situ hybrid plant cross fruit set percentage

There were different levels of crossing success for the reciprocal crosses determined by the ability of hybrid F1 seed to germinate, as depicted in Figure 2. The three generations of hybrid plants displayed varying fruit set percentages, defined as the number of pollinated flowers that developed fruit. The F1 fruit set percentage was relatively high at 56% with 30 of 53 pollinated flowers setting fruit (Table 4). The F2 fruit set percentage was lower at 5% with 3 of 66 crosses setting fruit (Table 4). As of March 2023, the F3 fruit set percentage was 0% with 0 of 6 flowers setting fruit (Table 4).

Table 4 Hybrid plant fruit set percentage.

Hybrid Generation	Crosses that result in fruit set (n)	Percent (%)
F1	30	56
F2	3	5
F3	0	0

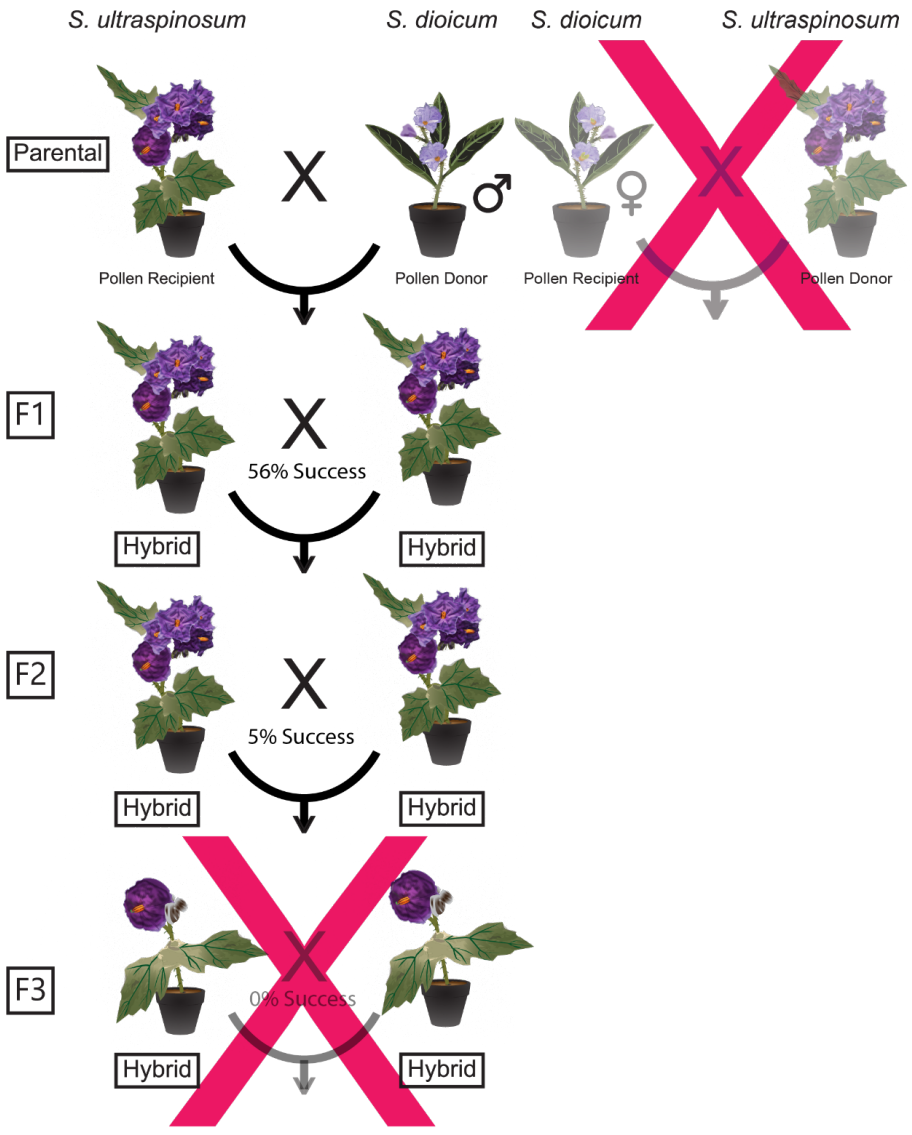

Figure 2 Experimental set up from the parental to the F3 hybrid generation noting crossing success. The red X indicates an unsuccessful cross measured in the parental generation as a failure of seed to germinate whereas for the filial generations a failure to set fruit.

Floral architecture and pollen morphology

Initial observations of the generations revealed different inflorescence composition across the generations. Inflorescence counts revealed that during a single snapshot of time, the F2 generation displayed 23 of 51 typical andromonoecious inflorescences, 10 of 51 aborted inflorescences, 12 of 51 inflorescences with the cosexual flower aborted but the staminate flower remaining, and 6 of 51 inflorescences with the staminate flowers aborted and the cosexual flower remaining (Table 5). The F3 generation displayed 0 typical andromonoecious inflorescences, 237 of 237 aborted inflorescences, 0 inflorescences with the cosexual flower aborted but the staminate flower remaining, and 0 inflorescences with the staminate flowers aborted and the cosexual flower remaining (Table 5).

Table 5 Snapshot of inflorescence floral composition in a single day. Counts of complete inflorescences and inflorescences with aborted flowers.

Generation	Total inflorescences counted (n)	Andromo-noecious	Aborted inflorescences	Only cosexual flowers aborted	Only staminate flowers aborted
F2	51	23	10	12	6
F3	237	NA	237	NA	NA

All individuals in the F1 hybrid generation resembled *S. ultraspinosum*, thus all F1 plants presented as andromonoecious with the typical inflorescence architecture: cosexual flower at the base, with male flowers above (Figure 3A). Among individuals in

the F2 generation, inflorescences were again mostly typical with a cosexual flower at the base and small staminate flowers above it (Figure 3B), although as the plants aged some of the inflorescence began to show signs of deviation from andromonoecious inflorescence architecture. Typical andromonoecious inflorescences were observed, as well as inflorescences in which all the male buds died, leaving only a cosexual flower (Figure 3F); and inflorescences in which the cosexual bud died, leaving only male flowers (Figure 3D). Additionally, there were a few instances of flowers in the male position that presented with its stigma extended past the anther pores, which I am referring to as thin-style cosexual flowers (Figure 3D). The observed style was noticeably shorter and thinner than the typical cosexual style (Figure 4B). F3 hybrids presented significant new variation as compared to what was observed in the F1 and F2 generations, although few buds appeared to display the normal andromonoecious architecture (Figure 3C). The majority of F3 inflorescence buds were aborted (Figure 4H). Those inflorescences with buds that were not aborted were almost completely cosexual, with nearly all staminate buds aborted (Figure 3E). Additionally, male buds appeared larger than typical Figure 3G). Flower growth was inadequate compared to previous generations, and the plants had a shorter and more compact growth habit. Current growth suggests that male flowers may be possible; however, it is currently believed that these male flowers are restricted to certain individuals. The individuals with all cosexual flowers also display the male position flower with its stigma protruding past the anther pores. Pollen evaluations across the generations revealed little variation in pollen type, and all pollen was porate.

Figure 3 The variation in inflorescence types observed across the hybrid generations. Normal andromonoecious inflorescences in the F1 (A), F2 (B) and F3 (C) generations. Short-style staminate position cosexual flowers in the F2 (D) and F3 (E) generation. Staminate buds aborted and a cosexual bud present in the F2 generation (F). Reduced cosexual bud and relatively large staminate bud in the F3 generation (G). Aborted staminate and cosexual buds in the F3 generation (H).

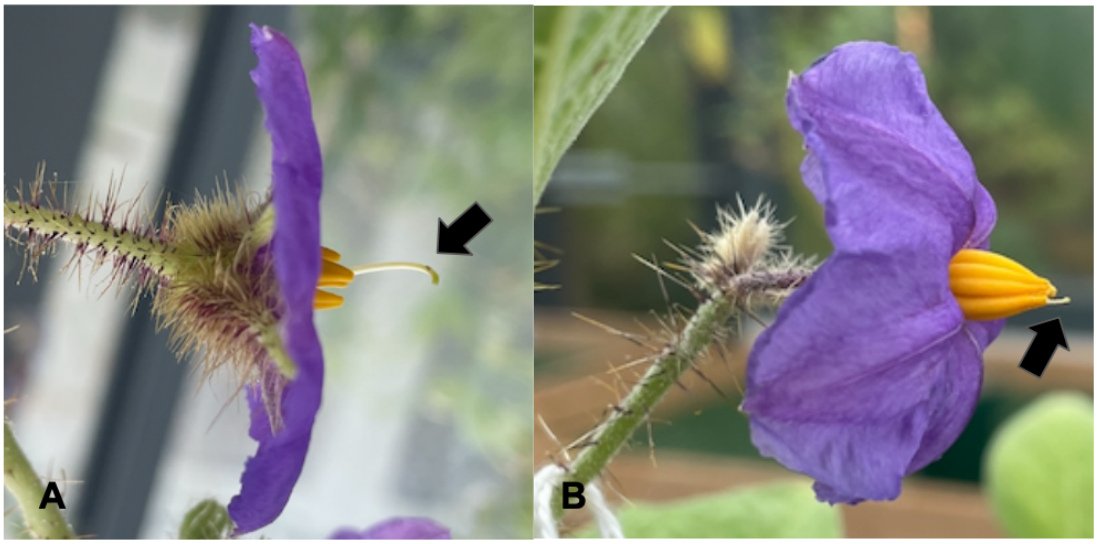

Figure 4 Comparison of style lengths in cosexual flowers. The arrow indicates the style. (A) Typical style length and width for a cosexual flower. (B) Thin-style cosexual flowers observed in the F2 and F3 generation. Notice the relative distance between anther and the tip of the style.

Initial observations of qualitative features of leaves indicated that leaf bases were oblique for all generations (Table 6 & Figure 5). Leaf margin was entire for *S. dioicum*, but lobed for *S. ultraspinosum* and all hybrids (Table 6 & Figure 5).

Table 6 Qualitative leaf characters.

Generation	Leaf base	Leaf margin
S. dioicum (paternal)	Oblique	Entire
S. ultraspinosum (maternal)	Oblique	Lobed
F1	Oblique	Lobed
F2	Oblique	Lobed
F3	Oblique	Lobed

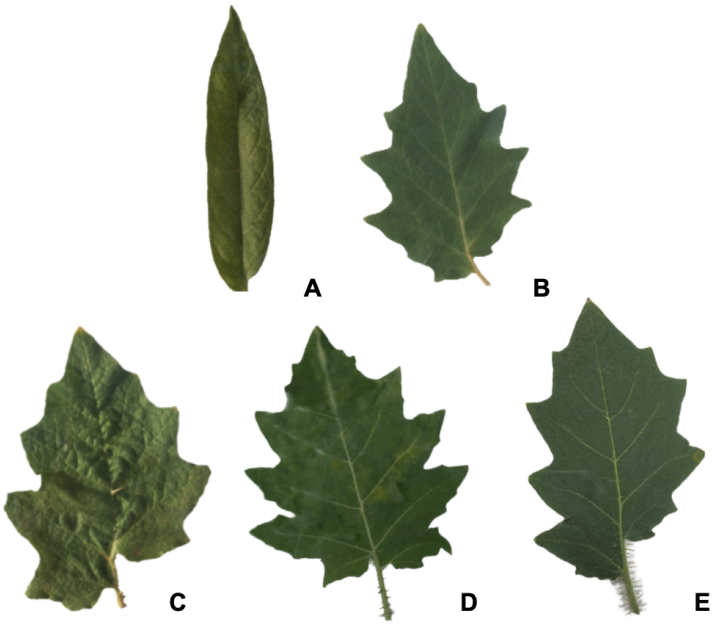

Figure 5 Image of leaf morphology for parental and hybrid offspring. Each leaf is representative of *S. dioicum* (A), *S. ultraspinosum* (B), F1 hybrids (C), F2 hybrids (D), and F3 hybrids (E).

Morphological measurements and analyses

Analysis of variance (ANOVA) indicated that the F3 generation differs significantly ($P<0.05$) from both the F1 and F2 generation in 4 of 8 examined vegetative characters (Figure 6). Of these four vegetative characters, which include height (Figure 6A), leaf length (Figure 6B), leaf width (Figure 6C), and leaf area (Figure 6D), the F3 generation also differed significantly from both parents, with three of those characters in the direction of *S. ultraspinosum*, while one character, height, was more consistent with the average height of *S. dioicum*. For one vegetative trait examined, upper leaf trichome

count (see Appendix Figure 11), the F3 generation did not differ significantly from either parent, while the F1 and F2 generation differed significantly from *S. dioicum* but not *S. ultraspinosum*. For one vegetative trait examined, lower leaf surface trichome count (see Appendix Figure 12), neither parental nor hybrid generations significantly differed.

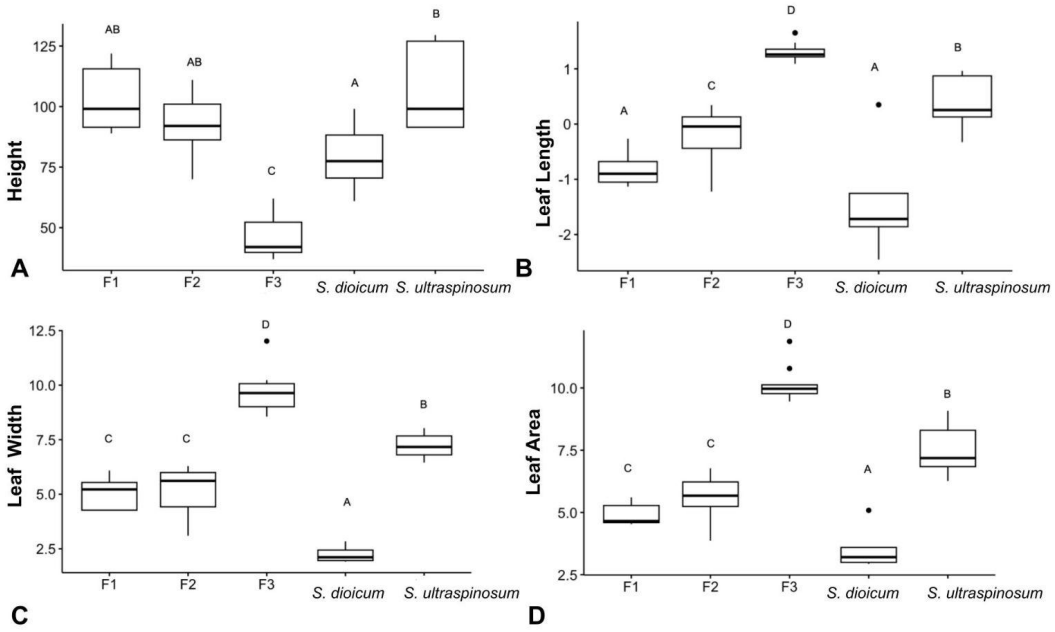

Figure 6 Boxplots for vegetative characters. Height (A), leaf length (B), leaf width (C), and leaf area (D) with letters indicating results of ANOVA and Tukey post-hoc analysis. Generations with the same letter did not significantly differ.

Of the ten floral traits examined, F3 measurements could only be conducted for two traits, petal length (Figure 7B) and anther length (see Appendix Figure 13), due to inadequate flower growth in the F3 generation at the time of measurement. Of these two floral traits (petal length and anther length), analysis of variance indicated that the F3 generation flowers measured did not significantly differ from either parent, while the F1

and F2 generation significantly differed from *S. dioicum* but not *S. ultraspinosum*. In six of ten floral traits measured, calyx length (Figure 7A), petal length (Figure 7B), corolla diameter, pedicel length, anther length, and style length, the F1 and F2 generations significantly differed from *S. dioicum,* but converged on the maternal *S. ultraspinosum* (see Appendix Figures 13-17). This was accounted for by either one or both generations of F1 and F2 hybrids not significantly differing from *S. ultraspinosum* or moving in the direction of *S. ultraspinosum*. Three floral traits (ovary width (Figure 7C), anther length, and ovary length) indicated that the F2 generation was moving in the direction of *S. dioicum*, with F2 ovary width differing significantly from *S. ultraspinosum* but not *S. dioicum*.

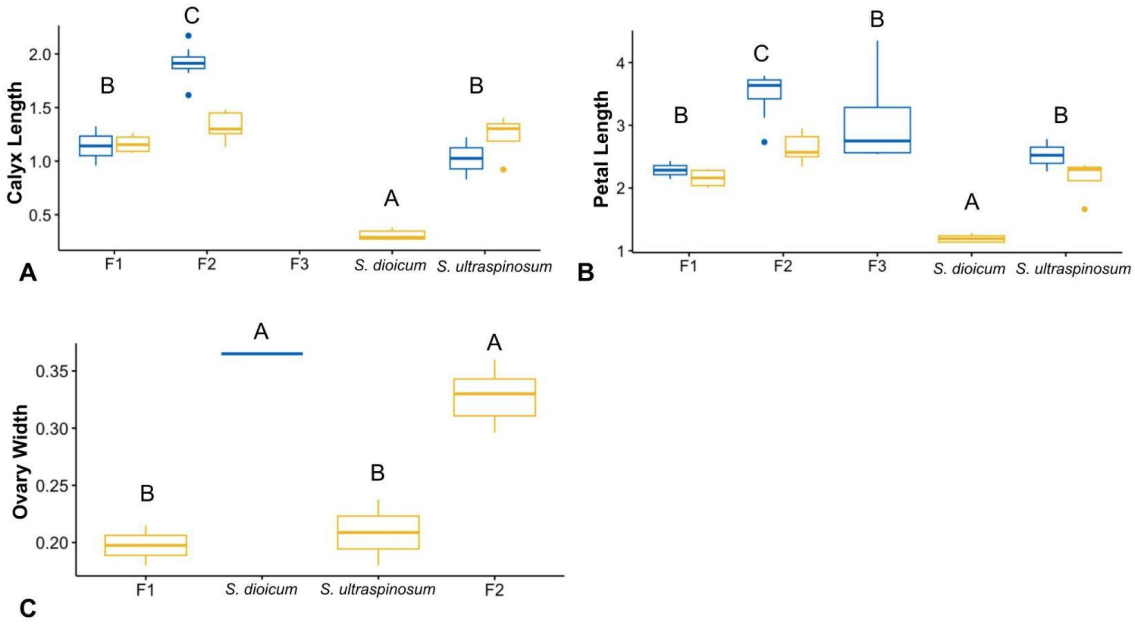

Figure 7 Boxplots for floral characters. Calyx length (A), petal length (B), and ovary width (C) with letters indicating results of ANOVA and Tukey post-hoc analysis. Generations with the same letter did not significantly differ. The blue box indicates cosexual flower values while the yellow box indicates staminate flower values.

PC1 and PC2 determined by the principal component analysis (PCA) described 75.3% of the total variation between the two parental species and their hybrids (Figure 8). The first principal component of the parental and hybrid generations, which was most highly correlated with floral and leaf traits, accounted for 48% of the total variance. The second principal component, which was most highly correlated with height, stem, and prickle traits, described 27.3% of the total variance. PC1 split the generations into three groups, with *S. ultraspinosum*, the F1, and the F2 generation clustering together; and the F3 generation and *S. dioicum* clustering independently. PC2 splits the generations into two groups with *S. ultraspinosum*, the F1, and the F2 generation again clustering together, and *S. dioicum* and the F3 clustering together.

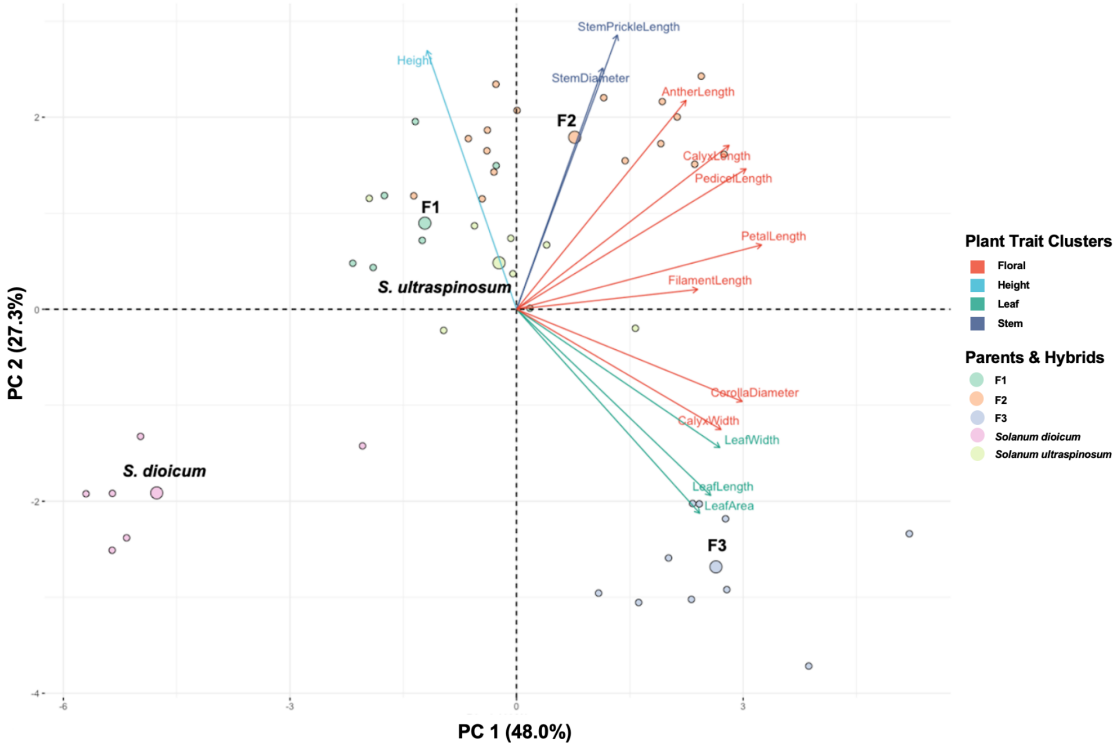

Figure 8 Principal component analysis (PCA) depicting variation in plant traits across generations. Showing loading plot (plant trait clusters) of hybrid and parental characters varying significantly by generation. The first two principal components explain 75.3% of the variation.

Fluorescence microscopy

The observed gynoecium in the F2 generation showed that pollen was largely successful at germinating (Figure 9), however the majority of pollen tubes were unsuccessful at reaching the ovary (Figure 9A, B). Few crosses were observed to lack pollen tube growth (Figure 9C). The single fluorescent cross conducted in the F3 generation was successful in reaching the ovary (Figure 9D and Figure 10).

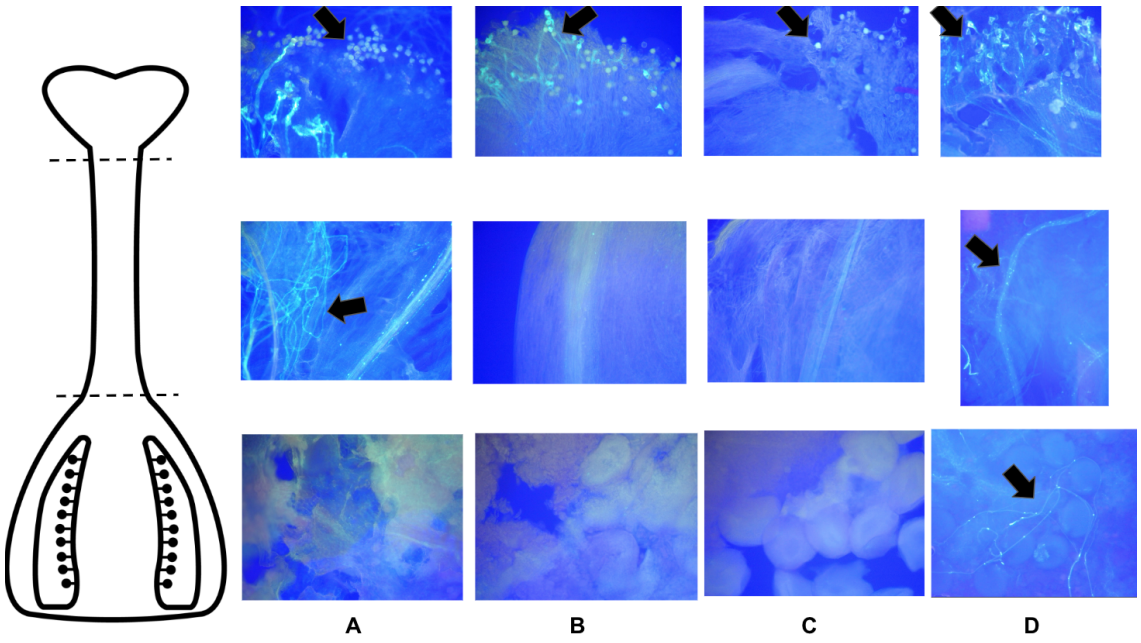

Figure 9 Fluorescence micrographs showing pollen tube growth in the gynoecium of F2 hybrids of *S. ultraspinosum* and *S. dioicum*. Gynoecium stained with aniline blue. The arrows indicate pollen or pollen tube growth. The first section represents the stigma of the recipient gynoecium, the second section represents the pollen tube, and the third section represents the ovary. The following crosses are depicted: (A) pollen tube abortion in the style, (B) pollen germination but lack of pollen tube growth down the style, (C) lack of pollen germination, and (D) a successful cross with pollen germination, pollen tube growth, and pollen tube growth into the ovary.

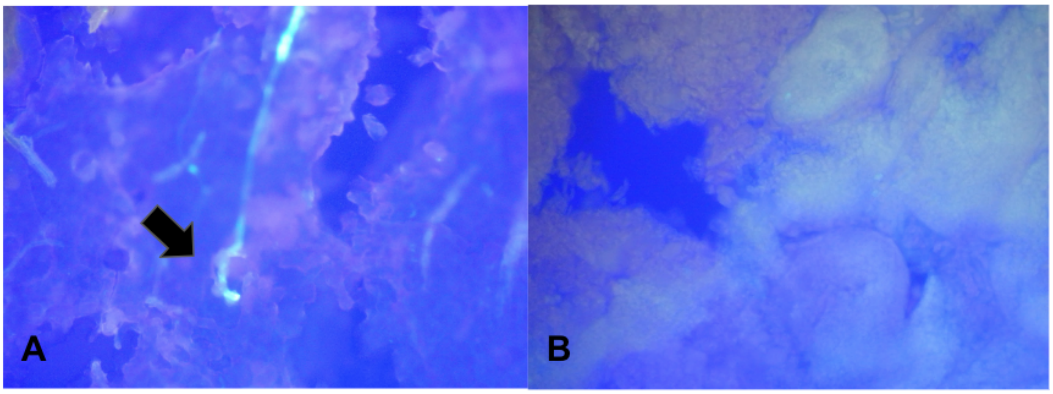

Figure 10 Fluorescence micrographs comparing the ovaries of successful (A) and unsuccessful (B) crosses. The section that brightly fluoresces is a pollen tube successfully reaching the ovary with an arrow to indicate it.

Evaluations of the gynoecium of F2 crosses viewed under fluorescence revealed that the majority of F2 crosses (16 of 21) did have pollen germination at the stigma and pollen tube growth down the style (Figure 9A, B; Table 7), although the tubes were aborted about one third down the ovary (Figure 9A). Additionally, three successful crosses were observed in the F2 generation, which accounts for the fact that some cross pollination attempts were successful, yielding the F3 generation (Figure 9D and Figure 10A; Table 7). Due to inadequate flower growth in the F3 generation, only one cross was able to be evaluated under fluorescence in the F3 generation. This single cross revealed successful pollen germination, as seen in the pollen tube growth through and into the ovary.

Table 7 Fluorescence microscopy crossing results

Pollen tube growth	Number of crosses	Percent (%)
No pollen germination	2	10
Pollen germination and pollen tube growth through style	16	76
Successful pollen tube growth to ovary	3	14

Discussion

Ex situ hybrid plant cross germination percentage

The F1 seed germination percentage was particularly important because it determined the parental role of contribution to the hybrids. While the study design used seeds with each parent in both roles as pollen donor and pollen recipient, the only successful hybrid seedlings from the original crosses were in one direction, with *S. dioicum* as the pollen donor and *S. ultraspinosum* as the pollen recipient due to the 0% germination rate in the reciprocal cross. The inability to grow successful seeds from the reciprocal cross may indicate hybrid seed incompatibility (HSI) caused by endosperm defects (Coughlan, 2022). Based on previous studies of HSI, it is reasonable to conclude that the successful cross relied on a larger ratio of endosperm gene contribution from the andromonoecious

pollen recipient parental species (*S. ultraspinosum*), thus incompatibilities may result from the larger genetic contribution of *S. dioicum* in the reciprocal cross. However, it should be noted that this is largely speculative due to the lack of genetic data. Another possible reason for the lack of seed germination in the reciprocal cross could be differences in seed size optimization between parental species (Dogra and Dari, 2019). Because *S. dioicum* is known to produce smaller seeds, it is possible that the seeds with *S. dioicum* as the pollen recipient were not large enough to capture the genetic information of *S. ultraspinosum*, thus resulting in the 0% germination percentage observed.

Additionally, a reduction in germination percentage was observed across the generations, with the F1 generation observed with the largest germination percentage (92.5%), the F2 being slightly lower (84.7%), and the F3 being the lowest (36%). The low germination rate observed in the F3 generation was consistent with initial observations of the fruit produced. Of three fruits produced as a result of crosses in the F2 generation, one fruit was reduced in size compared to the other two fruits, and the seeds from this fruit did not germinate. Considerations of the reduced germination percentage in the F3 generation will be discussed below in combination with considerations of fruit set percentage.

Ex situ hybrid plant cross fruit set percentage

A relatively large decrease in fruit set percentage was observed first from the F1 generation (56%) to the F2 generation (5%), and finally from the F2 generation to the F3

generation (0%). The reduction in fruit set percentage constitutes a decrease in fitness and thus is indicative of a hybrid breakdown. One possible explanation for the decrease in fruit set percentage that is commonly used to explain hybrid breakdown is that a form of genetic incompatibility arose due to the fact that these alleles have not yet interacted and are being selected against, which was observed limiting the viability of seeds as early as the F1 generation in the reciprocal cross (Burton et al., 2013).

The observation of hybrid vigor in the hybrid generations, observed by improved vegetative and floral growth compared to the parental generations, is particularly compelling given the lack of successful fruit set percentage. While the improved growth of hybrids would presumably result in a more abundant fruit set, the opposite was true. Although seed size was not examined, germination percentage indicates the reproductive viability of the seeds produced. Decreases in both seed germination and fruit set percentage across the generations demonstrates the competing optimization for seed size (a mechanism to ensure seed germination) and general fertility, measured by the number of seeds that were actually produced as result of a crossing event. This might show that while the hybrids are benefiting from the increase in heterozygosity of the hybridization event resulting in increased growth, crossing success is ultimately detrimentally affected, reflected by both reductions in seed germination and reductions in fruit set. This illustrates the tradeoffs that occur in nature; while it is beneficial for the hybrids to grow more abundantly, generations that cannot efficiently reproduce might have little success in the short term and may suffer in the long term from small population size unless environmental conditions are favorable. Evaluations of the gynoecium of F2 crosses

using fluorescent microscopy were intended to understand the lack of fruit set, specifically where in the gynoecium rejection occurred (see discussion below).

Floral architecture and pollen morphology

The type of pollen produced was of particular interest, since inaperturate (non-functional) pollen is a hallmark of functional dioecy in *Solanum* , and thus can indicate a shift in sexual system (Anderson & Symon, 1989). Inaperturate pollen occurs in morphologically cosexual flowers, rendering them functionally carpellate (Anderson & Symon, 1989). The observation of porate (functional) pollen indicates that the hybrid cosexual flowers are not functionally carpellate as might be expected in a transition to functional dioecy. The relative lack of staminate flower growth and the persistence of cosexual flower growth in the staminate position might suggest a potential reversion to cosexuality, although the resulting cosexual system produces too few flowers and no crossing success thus far, which renders the sexual system ineffective.

An alternative explanation emphasizes the importance of flower morphology. The presence of male-positioned flowers that are morphologically cosexual with styles that are shorter and thinner than traditional cosexual *Solanum* flowers may alternatively indicate sexual segregation (Levine & Anderson, 1986). One consequence of the thin-style cosexual flowers observed in the F3 generation might be higher rates of self-pollination, since the anthers are located so close to the stigmatic surface that pollen has a higher likelihood of making contact (Levine & Anderson, 1986). Thus, it is possible that

the resulting cosexual flowers may be less functional as carpellate (pollen receiving) and more operational as staminate (pollen donating). If these thin-style staminate flowers are limited to one individual, then it is possible that the changes of morphological characters are indicative of a state of sexual segregation, or a sexual system closer to dioecy.

Morphological measurements and analyses

The analyses of variance for vegetative characters indicated that the F3 generation is morphologically distinct from both F1 and F2 generations. This difference is especially apparent for height, where the F3 generation plants noticeably displayed a more compact growth habit compared to all other generations and display hybrid vigor despite inadequate flower development. The analyses of variance for floral characters that could be conducted for the F3 generation, petal length and anther length, indicated that the F3 generation was diverging from the previous two generations and displaying a phenotype that represented a possible intermediate between both parents, as opposed to being influenced by only *S. ultraspinosum* as the F1 and F2 generation were for these traits. This indicates that the genetic influence of *S. dioicum* may be becoming more apparent in the F3 generation.

The majority of F1 and F2 floral traits converged on the maternal form (*S. ultraspinosum)* according to the ANOVA. This is consistent with previous studies that observed a maternal effect in *Solanum* (Hayes et al. 2018). However, this was not true for all floral characters. The ovary measurements for the F2 generation appear to be

diverging in the *S. dioicum* direction, contrary to the previous F1 generation, which converged on the maternal form. Ovary width in the F2 generation, in particular, significantly differed from *S. ultraspinosum* but not *S. dioicum*. This is interesting considering that ovary measurement comprises one of the most important female functional characters, and *S. dioicum* is known to display a wider ovary compared to *S. ultraspinosum*.

According to morphology there are three distinct groups in which *S. ultraspinosum*, the F1 and the F2 generation cluster together, followed by a separation of the F3 generation, and *S. dioicum* as depicted by the PCA. This is a strong indication that the F3 generation may be the first generation to display a drastic change in phenotype independent of the maternal effect previously observed in the F1 and F2 generation. The fact that PC1 was more correlated with floral and leaf traits is consistent with ANOVA results, in which the leaf trait values for the F3 generation significantly differed from both hybrid and parental generations. The fact that PC2 was more correlated with height, stem and prickle characteristics is also consistent with ANOVA results, in which the F3 generation height, stem, and prickle measurements were converging in the direction of *S. dioicum*, although the resulting difference may not have been statistically significant.

Fluorescence microscopy

Investigations of pollen tube growth were intended to better understand the lack of crossing success in the F2 generation. Since there was observed pollen tube growth

through the gynoecia of F2 and the single F3 hybrid generations, this suggests that the pollen was likely functional. Therefore, it is plausible that pre-zygotic genetic incompatibilities may be responsible for the failure of the cross. The single fluorescent cross conducted in the F3 generation was successful in reaching the ovary, contrary to what was expected based on the crossing results. Yet, due to the small sample size it is difficult to draw definitive conclusions. It is possible that, if left to develop, this cross would have resulted in fruit. However, due to the few crosses that were able to be conducted, it is not possible to say at this time.

Implications

While the anticipated result was the loss of pollen functionality in cosexual flowers leading to a functional separation of sexes in the hybrid progeny, the observation of a reversion to cosexuality from parents with andromonoecious and dioecious sexual system suggests that hybridization is an important mechanism of sexual system changes, although its role in the transition to dioecy is inconclusive in these species. This also suggests the fluidity of sexual systems—that while andromonoecy as an intermediate may be possible, a return to cosexuality may be an unexpected consequence. Due to the variation in cosexual inflorescences and the persistence of staminate buds in the F3 generation despite their inability to grow, it is difficult to definitively place these hybrids in one sexual system. The persistence of staminate buds might suggest a retention of sexual allocation, but the investment is ultimately futile as most buds aborted. The

presence of F3 individuals with flowers that were either mostly male or mostly cosexual may also indicate dioecy, although functional dioecy is not yet established due to the presence of porate pollen.

In light of the long-held view of dioecy as an evolutionary dead end, the resulting reversion to a system more closely resembling cosexuality is compelling and consistent with more recent findings. Perhaps one feature of *Solanum* species that make such a reversion possible is the retention of vestigial gynoecium in staminate flowers. Indeed, the retention of a diminutive gynoecium calls into question the definition of staminate in *Solanum* species—while the egg cell is not functional in staminate flowers, the flowers clearly retain the potential for gynoecium growth. Future studies may analyze the importance of this vestigial gynoecium—an important feature of *Solanum* flowers.

Future directions

Based on previous hybrid animal studies that cite mismatches of the mitochondrial and nuclear genomes contributing to late hybrid breakdown, it is possible that maternal backcrosses with *S. ultraspinosum* may restore hybrid fitness in the F3 generation (Burton et al., 2013). Additionally, determinations of hybrid ploidy may also offer insight into cytological differences between hybrids.

Work that is currently underway includes continuing to examine F3 generation growth, conducting more pollination attempts, and growing additional plants of the previous generations to replicate the results with larger sample sizes. A similar study with

hybrids of different *Solanum* species would also be interesting given the fact that the reciprocal cross was not possible.

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Appendix

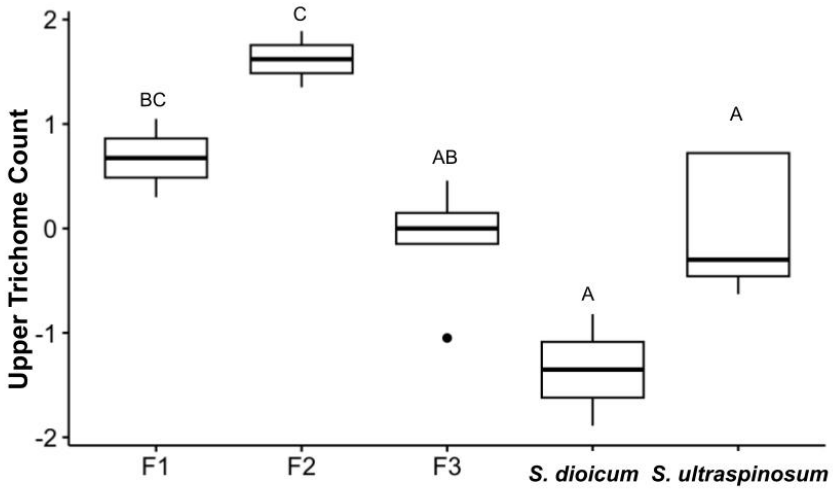

Figure 11 Boxplot for upper trichome count with letters indicating results of ANOVA and Tukey post-hoc analysis. Generations with the same letter did not significantly differ.

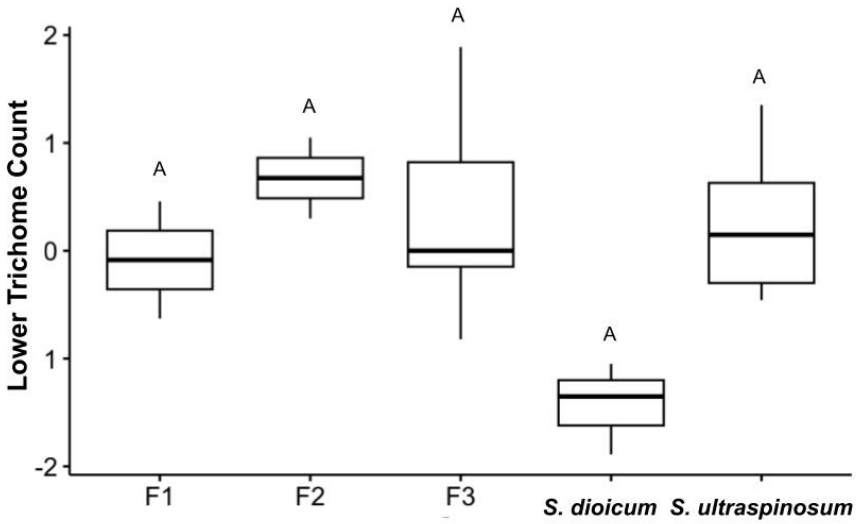

Figure 12 Boxplot for lower trichome count with letters indicating results of ANOVA and Tukey post-hoc analysis. Generations with the same letter did not significantly differ**.**

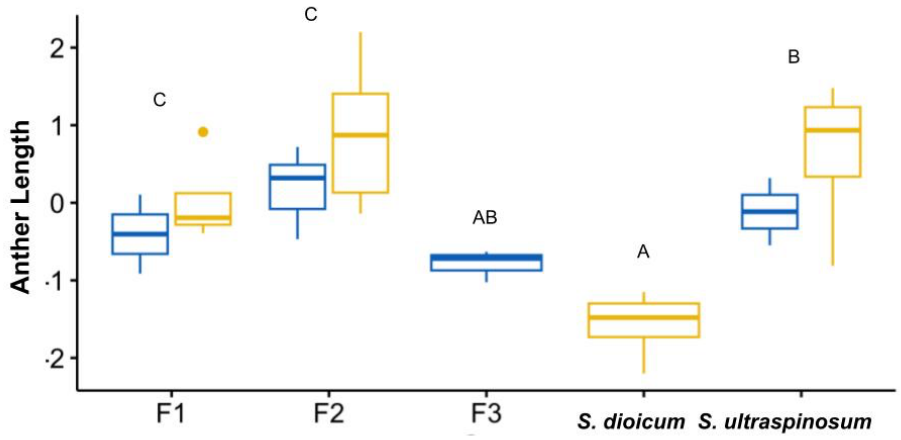

Figure 13 Boxplot for anther length with letters indicating results of ANOVA and Tukey post-hoc analysis. Generations with the same letter did not significantly differ. The blue box indicates cosexual flower values while the yellow box indicates staminate flower values.

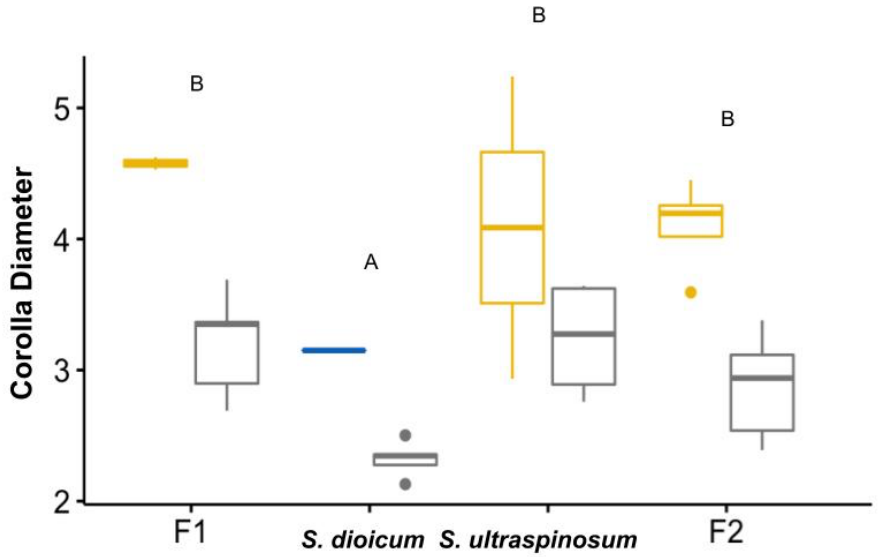

Figure 14 Boxplot for corolla diameter with letters indicating results of ANOVA and Tukey post-hoc analysis. Generations with the same letter did not significantly differ. The blue box indicates carpellate flower values while the yellow box indicates cosexual flower values, and the gray box indicates staminate flower values.

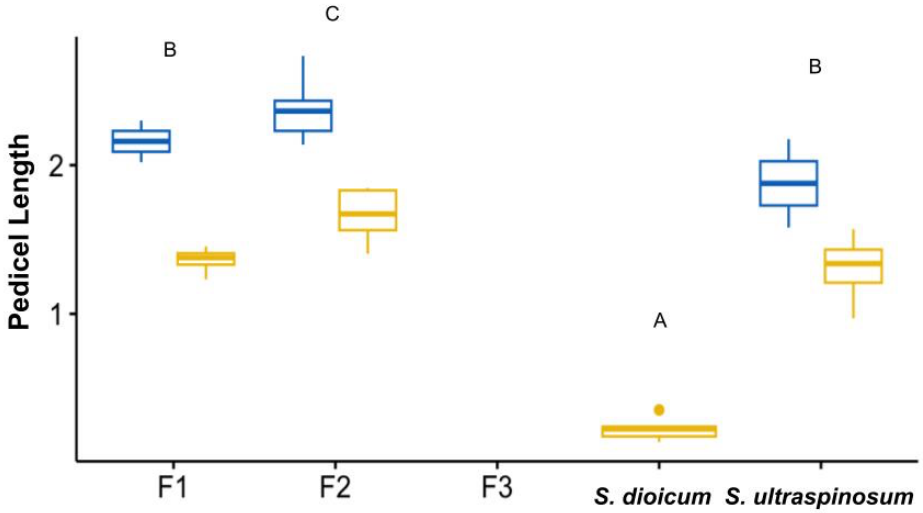

Figure 15 Boxplot for pedicel length with letters indicating results of ANOVA and Tukey post-hoc analysis. Generations with the same letter did not significantly differ. The blue box indicates cosexual flower values while the yellow box indicates staminate flower values.

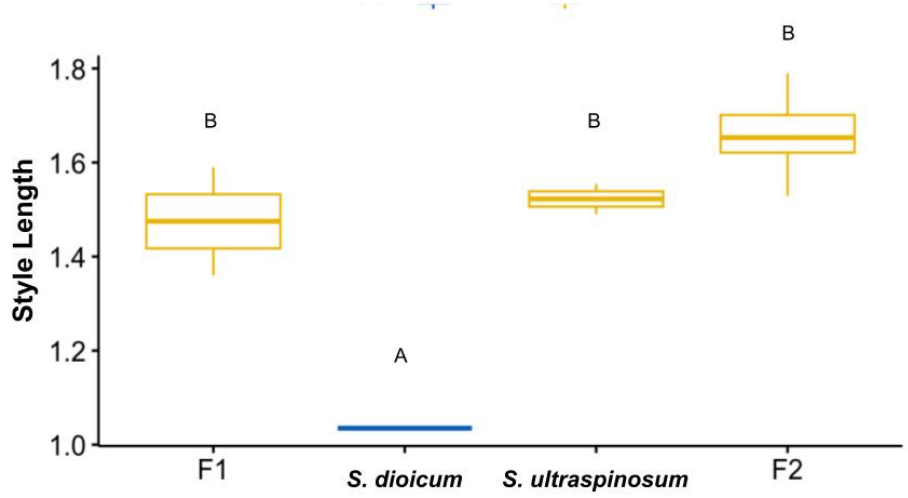

Figure 16 Boxplot for style length with letters indicating results of ANOVA and Tukey post-hoc analysis. Generations with the same letter did not significantly differ. The blue box indicates cosexual flower values while the yellow box indicates staminate flower values.

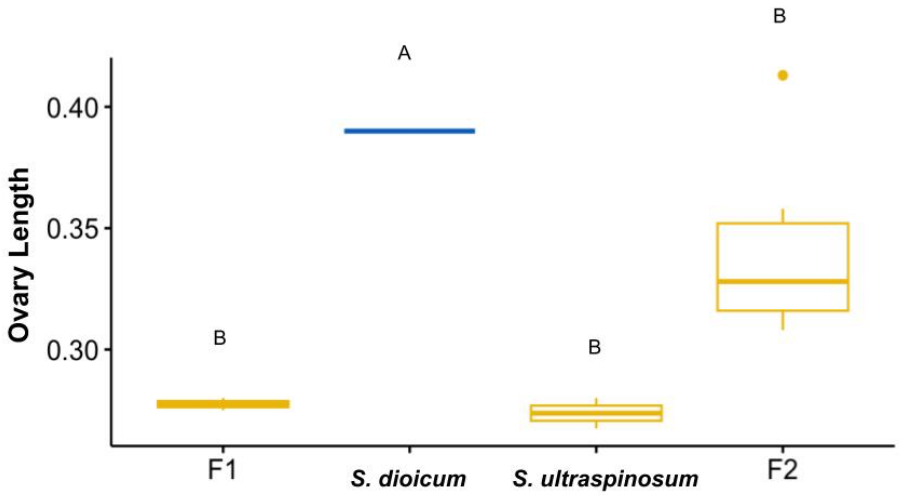

Figure 17 Boxplot for ovary length with letters indicating results of ANOVA and Tukey post-hoc analysis. Generations with the same letter did not significantly differ. The blue box indicates cosexual flower values while the yellow box indicates staminate flower values.