ASSESSING THE POTENTIAL EFFECTS OF SEED INCREASE AND INTERSPECIFIC HYBRIDIZATION ON GENETIC DIVERSITY AND FITNESS OF *COREOPSIS LEAVENWORTHII*

By

SARAH MAGEN SMITH

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2011

I did it, Gram!

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Zhanao Deng, for his guidance throughout my research project. I would also like to thank my committee members, Dr. David G. Clark, Dr. Kenneth H. Quesenberry, Dr. Jeff G. Norcini and Dr. Fred G. Gmitter, for advising me in their expertise of plant breeding, genetics and physiology. I would like to thank Joyce Jones, Gail Bowman, James Aldrich, Li Gong, Rick Kelly, Tomas Hasing, Mary Derrick, Monica Raguckas and David Czarnecki for assisting me with my research. At last, I would like to thank the Florida Wildflower Foundation, Inc. for funding this research.

TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

ASSESSING THE POTENTIAL EFFECTS OF SEED INCREASE AND INTERSPECIFIC HYBRIDIZATION ON GENETIC DIVERSITY AND FITNESS OF *COREOPSIS LEAVENWORTHII*

By

Sarah Magen Smith

August 2011

Chair: Zhanao Deng
Cochair: David G. Clark
Major: Horticultural Science

Coreopsis leavenworthii, one of Florida's state wildflowers, is nearly endemic to Florida and highly desirable for highway beautification and ecological restoration. Large-scale seed increase and planting of *C. leavenworthii* are becoming increasingly common in Florida. Previous studies have shown that potential genetic shifts and erosion may occur during seed increase, and natural hybridization can cause genetic contamination of produced seeds or planted populations. The objectives of this research were to 1) assess if phenotypic or molecular changes were occurring in *C. leavenworthii* over three successive generations at two locations, 2) assess the vegetative and reproductive fitness of synthetic F_1 and F_2 interspecific hybrids between *C. leavenworthii* and *Coreopsis tinctoria* and 3) determine the frequency and distance of pollen-mediated gene flow from *C. tinctoria* to *C. leavenworthii*. No significant differences or clear trends were detected between the increase and the original populations (G_0) in 12 morphological, physiological, reproductive and disease resistance characteristics of *C. leavenworthii*. Molecular marker analysis revealed subtle changes in SSR marker alleles and allele frequencies, slight decreases in the

total genetic diversity and slight increases in the genetic differentiation (G_{ST}) and genetic distances between the increase and the original populations. However, the original and the increase populations did not form any distinct clusters in principal component analysis, suggesting that the observed changes at the molecular level were not large enough to cause a significant genetic shift, and that the genetic diversity and integrity of the original population were maintained during seed increase.

Controlled pollinations showed that *C. leavenworthii* and *C. tinctoria* were fully compatible. Different vegetative and reproductive fitness traits responded differently to interspecific hybridization: number of days to flower was found to be affected by heterosis, plant dry weight expressed heterosis followed by hybrid breakdown, pollen viability decreased likely due to chromosome mispairing and seed production decreased likely by chromosome mispairing and dilution effects. Inheritance studies indicated that the maroon spot is controlled by a single dominant gene and is homozygous in *C. tinctoria*, making it a reliable morphological marker to detect pollen-mediated gene flow. The highest rate of pollen-mediated gene flow from *C. tinctoria* to *C. leavenworthii* was 4.24%, which occurred when the two species were grown at a 1.5 m distance, and the observed greatest pollen dispersal distance was 61.0 m. Two Hymenoptera species were identified as pollinators for both *Coreopsis* species. Overall, the current seed production practices seem to be appropriate for *C. leavenworthii* seed increase. Pollen-mediated gene flow could result in negative effects to *C. leavenworthii* and should be prevented to protect the genetic diversity and integrity of this narrow endemic species.

CHAPTER 1
LITERATURE REVIEW

Rationale

The concept of “planting natives” has become broadly accepted by our society and is often required by federal, state or local laws or policy. Exclusive native plantings have become a common practice in parks, forests, and natural areas, and increasingly common in roadside treatments (Rogers and Montalvo, 2004). One group of native plants that have been particularly popular is native wildflowers (forbs). Many states, including Florida, Idaho, Michigan, Ohio, Texas and Wisconsin, have adopted native wildflower planting programs for highway beautification. Use of native wildflowers along roadsides has not only increased aesthetic values, but also reduced maintenance costs, enhanced wildlife habitat and biodiversity, augmented soil erosion control and suppressed noxious weeds (Bryant and Harper-Lore, 1997).

Considering the mounting evidence of ecologically important phenotypic and genetic differences among populations within a species, native planting protocols often suggest using local provenances or ecotypes as genetically appropriate plant or seed sources (Rogers and Montalvo, 2004). It is expected that local ecotypes have developed adaptation, and local environments may favor local ecotypes’ flowering, seed production, soil seed bank development, seed germination and/or seedling establishment. Thus it is anticipated that local ecotypes may have better opportunities to become self-sustaining with appropriate management (Norcini et al., 2001, 2009).

The genus *Coreopsis* is Florida’s State Wildflower. *C. leavenworthii* is one of the 13 species of *Coreopsis* found in Florida (Gilman et al., 2007). This species is restricted to and ubiquitous in Florida (Kabat et al., 2007). Natural populations have been

documented in over 50 of Florida's 67 counties (Wunderlin and Hansen, 2004). The common habitats of this species include roadside ditches, wet pine flatwoods, and other moist disturbed sites (Kabat et al., 2007). Plants of this species can bloom year round in south Florida and form a dense flower cover over the foliage. For these reasons *C. leavenworthii* is very desirable for use in highway beautification projects.

The Florida Department of Transportation (FDOT) has used *C. leavenworthii* in a number of beautification projects and expressed strong interest to expand its current wildflower program and plant more local ecotypes of native wildflowers on roadsides, such as *C. leavenworthii*. Florida wildflower growers have collected seeds from natural populations and increased seeds to meet the demand (Norcini and Aldrich, 2004). It is anticipated that more seeds of *C. leavenworthii* will be produced by wildflower growers and used on more roadsides across the state.

In recent years, natural populations of *C. leavenworthii* in some areas of south Florida seem to be diminishing. This has led to a suspicion that the species' genetic diversity may be declining, due to factors such as habitat deterioration or destruction, roadside mowing practices and urbanization. Concerns have been also raised about the possibility that genetic shift and erosion may occur during seed increase. It has been suspected that certain genotypes of native plants may be better adapted to seed increase practices such as fertilization, irrigation and harvest date, and that these genotypes may produce more seeds than other genotypes (Rogers and Montalvo, 2004). Additionally, *C. leavenworthii* is sexually compatible with *C. tinctoria* (Parker, 1973; Smith, 1976, 1983). Although these two species are sexually compatible, the lack of pollen stainability and chromosome homology supports their species status (Parker,

1973). This species occurs naturally in a number of counties in Florida and has been used in highway beautification projects in Florida (Gilman et al., 2007; Parker, 1973; USDA, 2011b). There is real potential for *C. leavenworthii* and *C. tinctoria* to hybridize naturally and cause genetic contamination or swamping, which is the replacement of local genotypes as a result of a numerical and/or fitness advantage of immigrant genotypes. Thus there is a strong need to assess the effects of seed increase, interspecific hybridization and natural gene flow on the genetic diversity and genetic integrity of *C. leavenworthii*.

Genetic Diversity and Integrity

Significance of Genetic Diversity and Integrity

Genetic diversity is the variation in DNA among individuals and can change due to local adaptive pressures and other processes that can influence the mating success and survival of individuals (Hartl, 2000; Rogers and Montalvo, 2004). Along with mutation, genetic diversity provides the variation in a species' traits needed for adaptation to new conditions and establishing in new habitats. Genetic diversity is also the means for a species to become linked to other organisms such as pollinators, which may co-evolve with the species (Futuyma, 1979). Highly significant and positive relationships between the species' genetic diversity and fitness have been documented in many plants (Reed and Frankham, 2003). Genetic integrity refers to the quality and arrangement of genetic diversity in relation to natural processes that reflects changes in genetic composition caused by local natural selection and other processes that can affect mating and survival of individuals.

Conceivably, anything that compromises a species' genetic diversity and integrity will likely have cascading, negative effects on the species itself and associated

organisms (Havens, 1998). As such, and in face of accelerated changes in climate, maintaining the quality and quantity of genetic diversity has been considered as a critical component to the long-term survival of native plant species. However, it has been often very difficult to sense the urgency of such action because the loss of genetic diversity is often cryptic, the effects may not be obvious or visible for many years and genetic integrity can be severely degraded without an obvious loss of genetic diversity.

Natural Selection, Local Adaptations, and Ecotypes

The genetic diversity of a species is affected by several natural processes, including mutation, migration, genetic drift and selection (Futuyma, 1979). Natural selection is the best known of the processes affecting genetic diversity and is the only process that directly results in populations becoming better adapted to the environment. In response to natural selection from specific local or site-specific environmental conditions, such as temperature, day length, soil type and moisture level, certain populations of a species may become better adapted to the local environment (Booth and Jones, 2001). Such locally adapted populations have been recognized as ecotypes (Turesson, 1922). Different ecotypes may also differ in phenotypic characters, such as plant height, growth habits, leaf characters, earliness of maturity and reproductive habits (Grant, 1981). Genetically, different ecotypes may have different combinations of adapted genes and/or alleles.

In light of the existence of ecologically important phenotypic and genetic differences among populations within a species, native planting protocols have urged using local ecotypes as the genetically appropriate plant or seed sources (Rogers and Montalvo, 2004). The expectation is that local ecotypes are most adapted to local conditions and most tolerant of local stresses. Thus they will more likely become

established and self-sustained, which should result in reduced maintenance costs and better plant performance. Additionally, planting local ecotypes can help preserve the genetic diversity and integrity of native plants and preserve local pollinators, insects and other wildlife that have co-evolved with the plants of the local ecotypes and depend upon them for food and shelter.

Potential Changes in Genetic Diversity and Integrity during Seed Multiplication

Seed or plant increase is often required for large-scale planting of native species. Seed increase generally starts with collection of seed from natural populations followed by multiple generations of multiplication. This practice can make seed available in much larger quantities and result in lower costs of seeds. In the meantime, it protects native populations from over harvest. However, every stage of the increase process, from collection, planting location, production methods, harvesting practices to seed storage, can potentially cause unintentional genetic shifts in traits and erosion of genetic variation (Knapp and Rice, 1994; Meyer and Monsen, 1993).

During collection, seed are often collected from a portion of the natural populations. Thus only a portion of the alleles in the natural populations will be captured in the wild collection (Ellstrand and Elam, 1993; Hartl, 2000). Loss of genetic diversity and genetic integrity may occur if the sampled seeds do not represent the level and structure of genetic diversity in the natural populations.

Seed dormancy and seed shattering can be important adaptive traits, but they may be selected against and lost unintentionally due to artificial cultivation and harvest practices (Cai and Morishima, 2002). Seed dormancy may be lost after several generations of seed increase. Harvesting seeds in a narrow time window may limit genetic variation for timing of flowering, while harvesting toward the end or beginning of

seed maturity can result in genetic shifts. Growth rate and the timing of flowering could also be subject to unintentional selection.

Seed increase in a single location for multiple generations can result in adaptation to the specific production location or production practices (Burton and Burton, 2002). When seed increase is done in a habitat different from the original habit, multiple plant characteristics may change significantly and even become genetically fixed, which can be detrimental to the populations being increased (Antonovics, 1976). To avoid unintentional natural selection, seed increase should be started from a sufficient number of individuals, using appropriate practices, and done at appropriate sites where pollinators are present (Havens, 1998).

Seed increase in a single location for too many generations can result in local adaptation (Burton and Burton, 2002). To minimize such kind of unintentional selection during seed increase, the National Resources Conservation Service (NRCS) allows a maximum of four generations of seed increase for cultivars, tested releases and selected releases. For source-identified seeds, the NRCS and Association of Official Seed Certifying Agency (AOSCA) allow unlimited generations of seed increase, if no genetic shifts or erosion occur (Rogers and Montalvo, 2004). It has been stressed that to maintain the genetic diversity and integrity of the natural populations being increased, the genetic diversity of the starting materials should be assessed and used as a reference, and the genetic diversity and integrity of increased populations should be monitored closely.

Other measures have been proposed to counterbalance the selection for local adaptation during seed increase. One proposed measure is to allow 1-5% of gene flow

between populations per generation (Ellstrand and Elam, 1993). The authors consider that a gene flow frequency of 10% or more may have a significant effect on fitness, whereas 1% or less will be of little concern.

Assessing Genetic Diversity and Integrity

Common Garden Studies

Genetic diversity and integrity can be assessed at different levels and using a number of ways (Hartl, 2000). Before molecular markers became available, the assessment of genetic diversity and integrity was primarily based on the examination of phenotypic variation within populations (Berg and Hamrick, 1997). Phenotypic variation is generally controlled by many genes and can be influenced by environmental factors to varying degrees. To minimize the influence of environmental conditions on phenotypic variation, a “common garden” experiment design is often used (Berg and Hamrick, 1997; Clausen et al., 1940). In common garden studies, individuals of a population are grown in one or more common environments (Rogers and Montalvo, 2004). This approach is necessary and still commonly used. When assessing characteristics to determine if there is a loss of genetic diversity, characteristics of the entire life cycle should be evaluated (Edmands, 2007).

Molecular Markers to Assess Genetic Diversity

Since the mid-1960s, a number of biochemical and molecular techniques has become available (Harris, 1966). These techniques have allowed identification of alleles at many gene loci. Molecular markers represent discrete genetic differences and are generally insensitive to environmental changes, plant development and physiological changes (Hartl, 2000). Thus, molecular markers have become a valuable approach for assessing genetic diversity and integrity (Weising et al., 2005).

Many different types of molecular markers have been used to assess the genetic diversity within and among populations, including allozymes, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter-simple sequence repeats (ISSR) and simple sequence repeats or microsatellites (SSRs) (Weising et al., 2005). AFLP and SSR markers have been the most widely used markers in genetic diversity and integrity assessments (Mueller and Wolfenbarger, 1999).

AFLP is a useful marker to determine the genetic diversity among individuals and populations (Mueller and Wolfenbarger, 1999). AFLP allows for the screening of numerous DNA regions distributed throughout the genome. It has been reported that AFLP markers have the widest application when analyzing genetic variation within and among populations, which can help determine conservation steps needed for a species. When producing AFLP markers, careful marker design is very important for successful PCR amplification, especially for the adapters (Vos et al., 1995). Selectivity of the markers is acceptable with three selective nucleotides but is lost with four selective nucleotides. The number of amplified fragments may be dependent on the rare cutter enzyme and number of selective bases.

There are several benefits for using AFLP markers, such as DNA samples of any origin or complexity can be used because AFLP has the ability to assess numerous loci at one time making this marker system very efficient and it reveals many polymorphic bands with a small quantity of DNA (Blears et al., 1998; Mueller and Wolfenbarger, 1999; Vos et al., 1995). This marker system is reliable, reproducible, easy, affordable and fast to generate. The number of markers produced is limitless based on the

different combinations of restriction enzyme combinations available and the number and combinations of selective nucleotides, allowing for minor genetic differences to be detected. There is no need of prior knowledge of the DNA of an organism before development or use and small amounts of DNA are needed that segregate in a Mendelian fashion (Mueller and Wolfenbarger, 1999). Variation is eliminated due to the high selectivity of AFLP markers.

The main problem with this marker system is that it is a dominant marker, which does not allow for homozygous alleles to be distinguished from heterozygous alleles and can be less useful than codominant markers (Blears et al., 1998; Mueller and Wolfenbarger, 1999). Even though AFLP is known to be highly reproducible, there are some issues affecting the reproducibility of this marker system, such as the DNA used must be of high purity for complete digestion by the restriction enzymes, which can result in altering banding patterns (Blears et al., 1998). If DNA quantities are too low due to dilution, the DNA sequences flanking the restriction enzymes will not be random for some restriction fragments and the banding patterns may be altered.

SSR markers are those that detect variation in the number of short repeat sequences that change in a high frequency and detect multiple alleles (Langridge and Chalmers, 2004). SSRs are developed from DNA regions that flank specific microsatellite repeats, called primer binding sites, and are amplified using PCR (Glenn and Schable, 2005). The primer binding sites are not well conserved among distantly related species and are the most variable in the genome. SSR markers are densely distributed throughout the genome, highly variable and possibly the most powerful genetic marker available (Goldstein and Pollock, 1997; Zane et al., 2002). SSRs show

a large amount of polymorphism because of length variation due to the occurrence of different numbers of repeats and are widely distributed in higher plants at about one SSR for every 50 kb and are the ideal genetic markers in plants (Morgante and Olivieri, 1993). They are inherited in a Mendelian fashion, codominant and useful for population studies.

Because SSR markers are not conserved, they usually have to be developed for each species, which can be time consuming to detect, target and isolate (Glenn and Schable, 2005; Goldstein and Pollock, 1997; Zane et al., 2002). They are commonly found in noncoding regions of the DNA where nucleotide substitutions are more common. SSR makers can have a high mutation rate and can be unstable due to subtracting or adding a small number of perfect repeats and can be caused by polymerase slippage caused by slip strand mispairing errors during DNA replication and by unequal crossover or gene conversion of DNA strands during recombination (Goldstein and Pollock, 1997; Li et al., 2002).

Genetic Diversity Statistics

Appropriate sampling of individuals is critical for obtaining reliable estimates of genetic diversity parameters. Sample sizes that are too small can lead to underestimation of the true genetic diversity. Numerous modeling studies have shown that a sample size of at least 30 individuals should be used in molecular studies for estimating genetic diversity and integrity (Berg and Hamrick, 1997; Sinclair and Hobbs, 2009). The number of individuals needed to be evaluated may be different based on the species, where population structure and pollination biology can influence the movement of alleles within and among populations.

Several parameters have been frequently used in molecular genetic diversity assessment, including allele frequency, expected heterozygosity, total genetic diversity and genetic differentiation. Allele frequencies provide the base for estimating the other genetic parameters. The most important assumption for estimating these parameters is that the populations analyzed are in Hardy-Weinberg equilibrium (Berg and Hamrick, 1997). As described in population genetics, Hardy-Weinberg equilibrium can be approached under the following conditions: populations undergoing sexual reproduction, random mating, non-overlapping generations, large population size, no migration, mutation or selection and equal gamete production.

With Hardy-Weinberg equilibrium assumed, expected heterozygosity (H_E) can be calculated from the observed allele frequencies (Berg and Hamrick, 1997). This parameter measures the genetic variation at the allele level and is often referred to as genetic diversity. The magnitude of H_E depends on the proportion of polymorphic loci, the number of alleles per polymorphic locus and the distribution of allele frequencies within the population. Total heterozygosity (H_T) is the sum of the mean genetic diversity within populations plus the genetic diversity among populations. The G_{ST} value is an estimate of the genetic diversity that resides among populations, i.e. the genetic differentiation or divergence among populations.

Interspecific Hybridization

Occurrence of Natural Interspecific Hybridization

Natural interspecific hybridization has been known to be widespread between many plant species (Arnold, 1996). Compatibility between species is one of the main factors determining the frequency of natural hybridization. For interspecific hybridization to occur, cross pollination must occur between two species that are flowering at the

same time and are growing close enough for a vector to transfer pollen followed by successful fertilization that develops viable seeds that germinate and develop into a viable hybrid plant.

Short-term Effects

The degree of interspecific hybridization between plants varies depending on the location and species, which also affects the level of compatibility, where few hybrids can be produced or two species can evolve into one species (Ellstrand, 2003b). Natural hybridization within plants, where a single hybridization event can result in numerous evolutionary lineages, can be affected by the environment (Arnold, 1996). Typically, there is variation within a population between individuals' ability to survive and produce offspring due to selection that can be caused by genetic drift (Futuyma, 1979).

The fertility of interspecific hybrids depends on the species and the environment where they grow (Arnold and Hodges, 1995). Some interspecific hybrids are less fit than either parental species and can lead to nonviable F_1 progeny, F_1 hybrids that are viable but infertile or viable and fertile F_1 hybrids. Typically interspecific hybrids have a degree of sterility but are rarely completely sterile, where in a population of interspecific hybrids, some hybrid genotypes have higher fitness than either parent and even those with lower fitness can have ecological consequences (Arnold et al., 1999; Ellstrand, 2003b; Ellstrand et al., 1999). Most interspecific hybrids are intermediate between the parent species and the progeny produced, typically from backcrossing, from these parents typically resemble one of the parental species (Stebbins, 1959).

Even if the first generation of hybridization produces hybrids with limited fertility, later generations of hybridization may become more fertile (Arnold and Hodges, 1995). The percentage of interspecific progeny formed from highly sterile to completely sterile

individuals may be small but can have a large effect biologically (Grant, 1981). Partially fertile interspecific F_1 hybrids can reproduce by selfing, sibcrossing or backcrossing that produces a second-generation progeny that can continue crossing with the original plants resulting in a hybrid swarm that is an extremely variable mixture of species and interspecific hybrids. If a hybrid is less vigorous than either parent species in a certain habitat, it is less likely to be able to reproduce and disperse in that habitat (Harper et al., 1961). On the other hand, if the hybrids are more adapted to a habitat than the parent species, the hybrids could thrive and closely related species would evolve together.

Hybrids that inbreed for further generations are more likely to become stabilized into a fertile species with characteristics that are intermediate between the parent species or resemble an excessive form of one of the parent species (Abbott, 1992). New species formed through introgression are expected to have reduced genetic diversity, which are expected to increase at certain loci that are very different in both parent species. Once interspecific hybridization occurs, the level of stability of the new gene complexes will depend on mechanisms preventing recombination and segregation.

Long-term Effects

The effects of natural hybridization can be considered negative when rare species are lost resulting in outbreeding depression or genetic assimilation (Arnold, 1996). The negative connotation of interspecific hybridization stems from the fact that most interspecific hybridizations result in reduced fertility and viability. This is not true of all natural hybridizations and certain interspecific genotypes will have increased fitness from either parental species in certain environments, which could lead to adaptation into novel habitats or the displacement of either parental species in a habitat that is a better

fit for the hybrid genotype. Many would have a negative opinion of such a displacement, but others would argue that this could be the evolution of certain species. Natural hybridization can be a creative and ongoing process in evolution of numerous groups of organisms. Natural hybridization can have a positive effect when there is genetic enrichment of an endangered form of a rare species, increased genetic variability that can allow habitat expansion and using the hybrids to integrate beneficial phenotypes and genotypes back into the parental species.

Interspecific hybrids can exhibit increased fitness through heterosis. Heterosis caused by interspecific hybridization can lead to more fit progeny due to recombination of genes from both parent species, especially if the parents are inbred, particularly in environments well suited for the hybrids (Barton, 2001). Interspecific hybrids may be more fit than either parent species due to the hybrid population having more diverse genotypes, the ancestral lineage is reconstructed in the F_2 generation and fitness is determined by the interactions of the recombinant genes. Overall, hybrids formed from individuals of distinct environments will be less fit on average but some individuals with distinct genotypes will be more fit than either parent.

Generally, more divergent populations or species will create hybrids with lower fitness (Edmands, 2007). A decline in fitness due to outcrossing between distinct individuals or species is termed outbreeding depression and causes a significant decline in the F_1 hybrids relative to either parent or the mid-parent value and is environmentally dependent (Edmands, 2007; Ellstrand et al., 1999; Havens, 1998). There are two genetic mechanisms caused by outbreeding depression, dilution and hybrid breakdown (Hufford and Mazer, 2003). Dilution occurs after hybridization due to

the divergence of locally adapted genotypes that have diverged because of natural selection. Hybrid breakdown occurs from hybridization of individuals from distinct environments or from the mating of individuals with distinct gene complexes. Sterility of interspecific hybrids is usually due to genic differences or chromosomal rearrangements (Stebbins, 1959).

Pollen-mediated Interspecific Gene Flow

Introduction

Gene flow between closely related species occurs when species are sympatric (Harper et al., 1961). The effectiveness of breeding barriers, the chances of past migrations and the similarities of habitats where species grow can affect the frequency of gene flow. Animal and wind borne pollen dispersal usually follows a leptokurtic distribution, where a high frequency of gene flow occurs in a small range with little variance, where pollen is rarely distributed more than 1000 m and seed is rarely distributed more than 200 m with most gene flow occurring within 20 m of the source population (Ellstrand, 1992a; Levin, 1981). There are four main variables affecting cross pollination: the breeding system of the species, the isolation distance, plant density and the pollination vector (Bateman, 1947).

Potential Effects

The effects of gene flow depend on the amount and the level of success of gene flow between two species, which depends on the species, populations, individuals and years (Ellstrand et al., 1999). A small amount of interspecific gene flow can have a large impact on counterbalancing evolutionary forces, such as mutation, genetic drift and selection. Gene flow rates measured experimentally have typically exceeded 1% at distances of 100 m or more.

Interspecific gene flow is most problematic when two species interbreed significantly and are relatively compatible resulting in the production of a substantial amount of seed (Ellstrand and Elam, 1993). Another effect of gene flow can be caused by two distinct species growing in the same habitat and hybridizing freely causing the two populations to merge into one population and thus one species could "hybridize out of existence" (Harper et al., 1961).

Effects of Pollination Vectors and Population Size on Pollen-mediated Gene Flow

Most pollen collected by an insect is deposited on the next plant visited by the pollination vector, where the flight patterns of insects are random in direction (Levin, 1981). Usually there is pollen carryover for multiple plants, typically up to seven plants. The distances traveled by the vector are independent of the compatibility of the plants, seed viability and seedling survivorship. Pollen carry-over and direction of flight are dependent on floral reward and can affect pollen dispersal. Pollen deposition on a particular flower head is determined by the amount of time a pollinator spends on a flower, where more time will be spent at flower heads with a higher reward and there is a positive energy gain. This leads to less pollen carry over and gene flow in nectar rich populations.

Population size of both species greatly affects the potential rate of gene flow, where typically the gene flow rate increases as population size decreases because there are fewer targets in smaller populations (Ellstrand, 1992a, 2003a; Ellstrand et al., 1999; Ellstrand and Elam, 1993). Larger source populations and smaller sink populations can result in greater gene flow frequencies because the larger source populations will produce greater amounts of pollen and seed (Ellstrand et al., 1999; Ellstrand and Elam, 1993). Gene flow can disrupt local adaptation and cause extinction

of small populations depending on the degree of isolation and the frequency of successful hybridization with compatible species with greater effects in smaller populations (Antonovics, 1976; Ellstrand, 1992a). If the population size decreases rapidly, the rate and distance of gene flow can increase rapidly moving past the boundaries of a population, which can cause swamping effects to be substantial (Antonovics, 1976). Foraging pollen vectors will spend more time in a large population compared to a small population and will fly farther distances when plant density is low, potentially causing a greater swamping effect and a concern for plant conservation (Antonovics, 1976; Ellstrand, 1992a; Ellstrand et al., 1999; Levin, 1981).

Preventing Undesirable Pollen-mediated Gene Flow

To prevent unwanted gene flow into a population, pollinators and flowering times can be managed, immigrant pollen can be intercepted by planting other host populations around the population of interest or total eradication of the threatening species and interspecific hybrids (Ellstrand, 1992a). Some species prevent interspecific hybridization through several forms of reproductive isolation, such as pollen competition (Coyne and Orr, 1998). The heterospecific pollen tubes do not grow as quickly as the conspecific pollen tubes greatly decreasing the amount of interspecific seed produced. Generally 50 m or more is a great enough distance to prevent gene flow between two species but high outcrossing species can require about 500 m (Ellstrand and Elam, 1993). Gene flow can extend its range until it is stopped by barriers to dispersal such as climate, predators, competitors and resource availability (Slatkin, 1987). Strong barriers to prevent pollination between species can be difficult to produce if the species is constantly evolving (Ellstrand, 2003a).

An Overview of *C. leavenworthii* and *C. tinctoria*

The Genus *Coreopsis*

The genus *Coreopsis* is a member of Asteraceae, one of the largest families of flowering plants (Heywood, 1993). Members of this family have a variety of growing habits and can be found worldwide, except the Antarctica mainland. The family has been split into subfamilies and then further into tribes. *Coreopsis* belongs to Asteroideae subfamily and Coreopsideae tribe (Heywood, 1993; Ryding and Bremer, 1992). This genus consists of 8 sections; one of the most advanced sections is Calliopsis (Smith, 1975, 1983). *Coreopsis* is distributed throughout the Americas, the near Pacific Islands and Africa (Smith, 1976). The genus *Coreopsis* contains about 55 species that are distributed across the United States, thirteen of which grow in the state of Florida, including *C. lanceolata*, *C. grandiflora*, *C. tinctoria*, *C. basalis* and *C. leavenworthii* (FDOT, 1995, 2006; Gilman et al., 2007; Norcini, 2002; Sherff, 1955). Eleven species of *Coreopsis* are considered native to Florida, including *C. leavenworthii* (Gilman et al., 2007).

C. leavenworthii and *C. tinctoria*

Both species belong to the Calliopsis section and are known to be outcrossing species that are insect pollinated (Clewall, 1985; Wunderlin, 1998). *C. leavenworthii* has been reported to be growing in most counties in Florida and has been reported in only one other state, Alabama, and is considered endemic to Florida (Gilman et al., 2007; Parker, 1973; USDA, 2011a). *C. leavenworthii* is an annual to short-lived perennial with bright yellow ray flowers with a dark brown center, which are the disk flowers, that flowers from late spring in north Florida and any time in south Florida

(Gilman et al., 2007). *C. leavenworthii* seed is solely produced in Florida (Norcini and Aldrich, 2007).

C. tinctoria is an annual forb that overwinters as a rosette and germinates in late summer or fall (Whitten, 2002). The ray flowers are yellow with a red-brown portion close to the disk flowers, which are dark brown. This species grows in many soil types and best in full sun. *C. tinctoria* has been reported to be growing in most of the United States, including six counties in Florida but is not considered native to Florida (Gilman et al., 2007; Parker, 1973; USDA, 2011b). *C. tinctoria* is used mostly for landscape beautification that can be used in gardens, naturalized prairie or meadow plantings and along roadsides (Whitten, 2002).

Phenotypic and Molecular Diversity in Natural *C. leavenworthii* Populations

Because the nectar rewards of Asteraceae are small, plants within a population must be close in proximity and pollen dispersal will usually remain within that population because bees can probe many florets quickly and maintain a fairly high rate of energy intake (Price, 1997; Schmitt, 1980). In general Asteraceae must rely on long distance seed dispersal for the intermixing of populations and to keep genetic diversity levels high. Czarnecki et al. (2007) evaluated the phenotypic variation in natural populations of *C. leavenworthii* from north, central and south Florida. Differences were found among populations in characteristics such as growth habits, leaf type, days to flower, ray flower color, ray flower diameter and plant survival. Together natural populations displayed a substantial level of genetic diversity. In principal component analysis, natural populations were grouped together typically based on the region of origin. Czarnecki et al. (2008) further used AFLP markers to assess the genetic diversity of natural populations of *C. leavenworthii*. Natural populations from each region, north,

central and south Florida, were found to have a high percentage of polymorphic loci (68.6%) and a high level of genetic diversity, with a total genetic value from all natural populations (H_T) of 0.309. The G_{ST} value for the natural populations was 0.226, indicating some genetic differentiation among populations but most genetic variation remained within populations. The natural populations typically clustered within their geographic region of origin probably due to isolation by distance.

Previously, Crawford et al. (1984) evaluated the genetic diversity of *C. leavenworthii* and two other sister species in the section Calliopsis using isozymes. The total genetic diversity in *C. leavenworthii* and *C. tinctoria* was 0.187 and 0.235, respectively. It was suggested that the difference in the number of populations and individuals sampled accounted for the difference in genetic diversity. *C. tinctoria* seemed to have a higher proportion of polymorphic genes, a greater number of alleles per polymorphic gene and more heterozygous loci than *C. leavenworthii*. The higher number of alleles per polymorphic gene was caused by a greater number of low frequency alleles at several genes in *C. tinctoria*.

When the mean genetic identities were compared within and among species, *C. leavenworthii* and *C. tinctoria* had similar values (Crawford et al., 1984). The within population genetic identities for *C. leavenworthii* and *C. tinctoria* was 0.966 and 0.958, respectively, and among the two species was 0.944. These parameters indicate that the two species were as similar within populations as between species. Archibald et al. (2005) found an internal transcribed spacer (ITS) distance of 0.110 between *C. leavenworthii* and *C. tinctoria*, indicating that these two species are similar to each other.

Genetic Diversity in *C. leavenworthii* Seed Increase Populations

The seed production populations evaluated originated in central Florida but were used for seed increase in northern and central Florida (Czarnecki et al., 2007, 2008). The seed production populations showed less phenotypic variation for each character assessed compared to the natural populations regardless of seed increase location, indicating that the genetic identity of the seed production populations was maintained over various generations of production. A low level of genetic differentiation was detected among the seed production populations using AFLP markers. The percentage of polymorphic loci (63.4 to 72.5%) was comparable to the natural populations and total genetic diversity (H_T) was 0.251.

Compatibility between *C. leavenworthii* and *C. tinctoria*

C. leavenworthii and *C. tinctoria* growing ranges approach each other in northwestern Florida, where there could be some infrequent intergradations between the two species (Smith, 1983). Twenty-nine F_1 hybrids were produced and grown in the greenhouse from crosses between *C. leavenworthii* x *C. tinctoria* var. *tinctoria* with an average of 31% pollen stainability (Parker, 1973; Smith, 1976). However, these F_2 progeny were stunted and did not reach maturity. The chromosome configuration in the F_1 hybrids consisted of 7 bivalents, 2 trivalents and 1 quadravalent, indicating at least two reciprocal translocations. Eleven F_1 hybrids were produced and grown from crosses between *C. leavenworthii* x *C. tinctoria* var. *similis* with 13% pollen stainability (Smith, 1976). Univalents, bivalents and trivalents were found at diakinesis of pollen meiosis, also indicating several structural differences in the genomes of the two species. Smith (1983) assessed the fertility of F_1 hybrids produced in the greenhouse from crosses between these species. F_1 progeny of the cross between *C. tinctoria* and

C. leavenworthii had a moderate pollen stainability of 30-60%, which is considered high enough to allow occasional gene exchange.

Research Objectives

C. leavenworthii is nearly endemic to Florida and highly desirable for native planting in highway beautification projects. Large-scale seed increase and planting of *C. leavenworthii* are becoming increasingly common in Florida. Previous studies have shown that potential genetic shifts and erosion may occur during seed increase and natural hybridization can result in gene flow between species and cause genetic contamination of produced seeds or planted populations. This study seeks to assess the potential of these genetic risks during *C. leavenworthii* seed increase and planting and to provide guidelines for minimizing such risks and maintaining the species' genetic diversity and integrity. The objectives of this study were to 1) assess if there were phenotypic changes or genetic shifts in *C. leavenworthii* occurring over successive seed increase generations in morphological, physiological and reproductive characteristics and determine if seed increase location has played a role in causing potential genetic shifts, 2) use SSR markers to assess potential genetic changes that might occur to *C. leavenworthii* populations during seed increase in central and northern Florida over three generations, 3) determine the fitness level of *C. leavenworthii* and *C. tinctoria* interspecific hybrids in the F_1 and F_2 generations by comparing them to intraspecific populations of *C. leavenworthii* and *C. tinctoria* and 4) determine the inheritance of morphological markers that could potentially be used to reliably detect pollen-mediated gene flow, assess the effects of planting distance on pollen-mediated gene flow from *C. tinctoria* to *C. leavenworthii* and identify insect pollinators that may be involved with pollen-mediated gene flow.

CHAPTER 2
ASSESSING PHENOTYPIC CHANGES OF A NATURAL *COREOPSIS LEAVENWORTHII* POPULATION DURING SEED INCREASE

Justification

Coreopsis leavenworthii has only been recorded in Florida and two counties in Alabama. It is solely produced in Florida and commonly found along roadside ditches and highways, where it is used by the Florida Department of Transportation (FDOT) for highway beautification and erosion control (Norcini and Aldrich, 2007). Seed of *C. leavenworthii* is typically produced from a segment of a natural population for several generations. There are two major concerns for seed increase practices of this species, 1) to uphold genetic diversity from generation to generation and 2) to assess whether producing seed and growing populations in a different location from the natural population will affect the persistence and genetic diversity of the production population. Because a segment from a natural population is used for several generations of seed increase, there is the potential for a loss of characteristics, such as leaf type, seed production and flowering time, if the range of characteristics is not broad enough or representing all of the natural population to adapt to changing environmental conditions. The entire life cycle of the population should be assessed to accurately evaluate the phenotypic diversity of a population to ensure that the population was unaffected by the seed production practices (Edmands, 2007). When a population is moved into a new habitat, many traits are naturally selected for simultaneously, which could result in a phenotypic response that causes changes in the appearance of the individuals of a population (Antonovics, 1976). Because of this simultaneous natural selection, the fitness level of the population will depend on the correlation between the characteristics and this level of fitness can change with different habitats. The genetic diversity of the

natural and seed production populations should be assessed to be sure that the genetic diversity is upheld from generation to generation and in different locations. Typically seed of *C. leavenworthii* has been produced for several generations from the same natural population that has been used for re-establishment and restoration projects. A decrease in genetic diversity may result in inadequate establishment leading to poor plant performance and higher maintenance costs.

Several factors can affect genetic variation within and among populations, such as population size, geographic distribution, primary mode of reproduction, mating system, seed dispersal mechanism and community type of the species, where inbreeding depression can result from the same plants interbreeding, a rapid decline in population size or changes in vector preferences (Hamrick, 1983). If inbreeding depression occurs, the genetic diversity of the population may decrease causing inadequacy in yield or degradation of the species over successive generations. The size of the population being used for seed increase can have a large effect on inbreeding depression by having a larger effect on larger populations rather than smaller populations because the frequency of deleterious alleles already declined in smaller populations making inbreeding depression less profound (Thiele et al., 2010; van Treuren et al., 1993). For example, even though *Scabiosa columbaria* is an outcrossing species, it experienced severe inbreeding depression that was caused by limited gene flow between populations and could have led to extinction (van Treuren et al., 1993). Inbreeding depression caused a decrease in biomass production, root development, adult survival and seed set.

The most widely used approach to assess genetic changes during seed increase or production has been conducting common garden studies, where plants from seed increase are grown under common conditions and their characteristics are evaluated side by side. Simon and Kastenbauer (1979) used this design to evaluate the morphological traits and yield for timothy, meadow fescue and perennial rye grass that were produced in Germany and three locations in the United States. After seed increase of multiple generations, seed was sent back to Germany for evaluation. In another study, seed of three cultivars of Italian ryegrass was increased over two generations at one location in Japan and four locations in the United States and the populations were evaluated for genetic stability at one location in the United States (Rincker et al., 1982). Neither of these studies showed adverse effects from seed increase in multiple locations or over successive generations.

A previous study examining the morphological characteristics of *C. leavenworthii* natural and seed production populations revealed that there was a substantial amount of genetic diversity among the natural populations (Czarnecki et al., 2007). The populations from similar geographical regions were found to be more similar than those from different regions. The seed production populations, originally from central Florida and increased at different sites in Florida for one to four generations, remained genetically similar and maintained a moderate level of genetic diversity. Producing seed over successive generations and different locations could affect the genetic diversity of *C. leavenworthii*. In the previous study seed production took place at several different locations and not over successive generations. It can be difficult to compare these populations because phenotypic differences could have occurred

because of location, generation or both. To determine if location or generation has caused morphological changes or genetic shifts, seed increase should occur at the same multiple locations over successive generations.

In the current study, seed from a natural *C. leavenworthii* population in central Florida was increased simultaneously in central and northern Florida over three successive generations followed by common garden studies of the original and increased seed populations in central Florida, where individuals of several populations were grown in the same location for evaluation. The objectives of this study were 1) to assess if there were phenotypic changes or genetic shifts occurring over successive seed increase generations in 12 morphological, physiological and reproductive characteristics and 2) determine if seed increase location has played a role in causing potential genetic shifts. The information obtained will be very valuable for defining *C. leavenworthii* seed increase practices for maintaining the species' phenotypic diversity and integrity during seed increase.

Materials and Methods

Seed Collection from a Natural Population

Seed collected from a *C. leavenworthii* natural population in Reedy Creek Mitigation Bank in Polk County, FL (Lat. 27°51' N, Long. -81°41' W, USDA cold hardiness zone 9a, AHS heat zone 11) was used as the seed source for subsequent increases. This source was referred to as Generation 0 (G_0). The collection consisted of 1220 seed heads from 122 individuals randomly selected out of the natural population (10 seed heads per individual). The collection was made on 1 July 2006 from 75 plants and on 24 September from another 47 plants. The soil at the growing

site of the natural population was a mixture of ultisols, entisols and alfisols. Seed from the different individuals were combined and split into four seed lots.

Seed Increase

One seed lot from the natural population was maintained at the North Florida Research and Education Center (NFREC), Quincy, FL (Lat. 30°32' N, Long. -84°35' W, USDA cold hardiness zone 8b, AHS heat zone 9) in northern Florida and another was maintained at the Gulf Coast Research and Education Center (GCREC), Wimauma, FL (Lat. 27°45' N, Long. -82°13' W, USDA cold hardiness zone 9b, AHS heat zone 10) in central Florida. At each location, seed was increased successively for three generations (G_1, G_2 and G_3), starting in spring 2007, by growing ~100 plants from seed of a previous generation and harvesting mature seed heads from ten open-pollinated flowers from each plant. Harvested seed heads were dried indoors at each site and then stored at the GCREC's seed storage room (10°C and 50% RH). The seed increase population was designated by its generation and its site of increase (C for the central Florida site and N for the northern Florida site).

Seed increase in central Florida

G_0 to G_1C. Seed from G_0 was sowed on the surface of Vergro verlite container mix A (Tampa, FL) on 16 January 2007 and germinated in the greenhouse (29.4/23.9°C) under mist and a natural photoperiod. Seedlings were transplanted into 80-mL cell containers (Landmark Plastic Corp., Akron, OH) of Vergro verlite container mix A on 22 February and grown in the greenhouse (29.4/23.9°C) under a natural photoperiod for about four weeks. The plants were then transplanted into 3.2-L containers filled with Vergro verlite container mix A mixed with 8.6 g of 15N-3.9P-10K (5-6 mo. Osmocote, The Scotts Co., LLC, Marysville, OH) on 21 March and grown for another three weeks

in the greenhouse until they became established. Before the established plants began flowering, they were moved out of the greenhouse onto a greenhouse bench and grown for about six weeks before seed collection under a natural photoperiod. Ten seed heads per plant were collected on 14 and 27 June from 110 plants (23.2°C and 71.1% RH) (Table A-1).

G_1C to G_2C. Seed from G_1C was sowed on 8 January 2008. Seedlings were transplanted on 13 February. Plants were transplanted into 3.2-L containers on 21 March. Ten seed heads per plant were collected on 28 May from 82 plants, 9 June from 70 plants and 26 June from 61 plants (23.3°C and 70.8% RH) (Table A-1). All other growing conditions were the same as those used for the increase of G_0 to G_1C.

G_2C to G_3C. Seed from G_2C was sowed on 7 January 2009. Seedlings were transplanted into 31-mL cell Speedling flats (128-cell Speedling flat) (Sun City, FL) on 27 January. Seedlings were transplanted in Fafard 3B soil media (Anderson, SC) into 3.2-L containers on 23 February. Ten seed heads per plant were collected on 1-3 May from 120 plants, 20 May from 118 plants and 8 June from 105 plants (21.6°C and 72.7% RH) (Table A-1). All other growing conditions were the same as those used for the increase of G_0 to G_1C.

Seed increase in northern Florida

G_0 to G_1N. G_0 seed was sowed on 9 January 2007 on the surface of Metro Mix 200 (Marysville, OH) in a 6.5-L flat. The flats used for seed sowing were placed on propagation mats set at 21°C in a greenhouse, where the heater was set at 15.5°C. The seedlings were transplanted in 74-mL containers of Metro Mix 200 on 6 February and fertilized with 100 ppm of 15N-13.2P-12.4K (Miracle-Gro, All Purpose Plant Food, The Scotts Co., LLC, Marysville, OH) starting one week later. The plants were

transplanted in the field on 6 March and fertilized with 9 g/plant of 18N-2.6P-10.0K (8-9 mo. Osmocote, The Scotts Co., LLC, Marysville, OH). The field, with soil type Orangeburg loamy sand, was covered in black landscape fabric, where the plants were spaced on 30.5 cm centers and planted in a single 30.5 m row. A total of ten seed heads were collected from each of the 100 plants as the seed heads developed and matured from July through August.

G_1N to G_2N. Seed from the G_1N population was sowed on 1 January 2008. The seedlings were transplanted on 1 February. The plants were transplanted into the field on 6 March. Ten seed heads per plant were collected from 14 May through June from 100 plants. All other growing conditions were the same as those used for the increase of G_0 to G_1N.

G_2N to G_3N. Seed from the G_2N population was sowed on 7 January 2009. The seedlings were transplanted on 6 February. The plants were transplanted into the field on 9 March. Ten seed heads per plant were collected from May through June from 100 plants. All other growing conditions were the same as those used for the increase of G_0 to G_1N.

Assessing Phenotypic Changes

Experimental design

Common garden studies were conducted to assess potential phenotypic changes of *C. leavenworthii* during seed increase. Seed from the different increase populations were germinated and plants were grown in the same environment in central Florida. Plants were arranged using a randomized complete block design with 15 blocks and five individuals per block in 2009 and five blocks with 15 individuals per block in 2010.

A total of 75 individuals were grown and assessed per seed increase population in the field for both years.

Growing conditions of the common garden study

2009. Seed from each population was sowed on 2 July 2009 in Fafard 3B soil media into 330-mL containers in the greenhouse (29.4/23.9°C). One hundred seeds were put into each container with five replications per population. Seed germination was counted and seedlings were transplanted on 21 July into 31-mL cell Speedling flats with Fafard 3B soil media. One hundred-twenty eight seedlings were transplanted for each population. The plants were transplanted in the field on 3 September in a single row. The field was a mix of Myakka, Haplaquents and St. Johns sandy soil. The beds were raised 25 cm high, 71 cm wide at the top and 81 cm wide at the base. Plants were spaced 30.5 cm apart within plots that were 92 cm apart in rows with 152 cm between rows. The plants were fertilized with a total of 169 kg ha^{-1} of nitrogen, 37 kg ha^{-1} of phosphorus and 210 kg ha^{-1} of potassium throughout the year through drip irrigation.

2010. Seed from each population was sowed on 20 January 2010. The number of seedlings that emerged was counted and seedlings were transplanted on 1 February. Plants were transplanted in the field on 5 April. All other growing and field conditions were the same as 2009 except no fertilization was applied and the plants were irrigated through seepage irrigation.

Data collection

Data collected from the field included plant height (cm), plant dry weight (kg), leaf type, days to flower (DTF), disk flower size (DFS) (diameter, cm), whole flower size (WFS) (cm), petal lobing, degree of petal overlap (DPO), number of ray petals per flower head (NRP), the number of seeds produced per five seed heads, seed

germination (%) of seed collected from the field and powdery mildew severity (PMS). The tallest part of the plant was measured for plant height. After the plants were removed from the field, they were dried in a drying room (37.8°C and 20% RH) and weighed after at least three weeks of drying. Data were collected for 2009 from 3 September 2009 to 15 January 2010 and 19 April 2010 to 14 June for 2010. Leaf types were categorized on a 1 to 7 point scale, where 1 was a simple leaf and 7 was the most complex leaf (Figure 2-1). Leaf type data were taken 94 days in 2009 and 113 days in 2010 after transplanting. DFS and WFS were measured from five flowers each that were approximately at the same level of maturity. Petal lobing was rated on a 1 to 6 point scale based on the majority of flowers on the plant, where 1 was the most simple petal (no lobing) and 6 was the most complex petal (most and deepest lobing) (Figure 2-3). The DPO was rated on a 1 to 3 point scale, where 1 had petals that were oriented in a pinwheel fashion and not touching (G), 2 was where the outside of the petals were touching but not overlapping (S) and 3 was where petals were overlapping (O) (Figure 2-5). PMS was rated on a 1 to 10 point scale, 1=no infection, 2=1-10%, 3=11-20%, 4=21-30%, 5=31-40%, 6=41-50%, 7= 51-60%, 8=61-70%, 9=71-80 and 10=81-100% infection (Hausbeck et al., 2002). PMS was taken 113 days in 2009 and 120 days in 2010 after transplanting. Seed collected in the field in 2009 was sowed on 8-10 March 2010 for seed germination tests.

Statistical analysis

Significant differences were found for each trait evaluated using analysis of variance (ANOVA) using PROC GLM in SAS (SAS, 1997). Data were transformed using the Arcsine Square Root method for the seed germination tests. Based on the ANOVA test, further statistical testing was performed using the Tukey W Procedure for

mean separation analysis in SAS. Changes in genetic diversity between populations for each phenotypic characteristic measured were described using the standard errors calculated as in Tesfaye et al. (1991) and discussed in Foote (1997). Relationships among populations were determined using principal component analysis (PCA) using NTSYSpc (NTSYSpc, version 2.2 [Rohlf, 2005]).

Results

Plant Height and Dry Weight

There were significant differences found between years but not among populations for plant height (Table 2-1). The mean plant height of the G_0 individuals in 2009 was 72.3 cm (Table 2-2). The mean plant height of individuals of the six increase populations was 69.1 to 73.9 cm, or 95.6% to 102.2% of that of the G_0. The mean plant height value for the G_0 individuals in 2010 was 21.6% smaller (56.7 cm) than that in 2009. Plant height decreased by 17.4% to 25.3% (55.1 to 61.0 cm) for the individuals of the six increase populations. The mean plant height for the increase populations in 2010 was 97.2% to 107.6% of the mean height of G_0, which was similar to the 2009 results.

Standard errors can be used to describe changes in genetic diversity as in Tesfaye et al. (1991) and Foote (1997). The standard error of the plant height mean for the G_0 individuals was 0.77 and 0.73 cm in 2009 and 2010, respectively (Table 2-3). The standard errors of the plant height means for the individuals of the six increase populations were similar to G_0 in 2009, ranging from 0.70 to 0.95 cm, indicating no change in phenotypic diversity over seed increase. In 2010 there was a slight decrease in standard error (0.41 to 0.66 cm) for the plant height mean for the six seed increase

populations, indicating a slight decrease in phenotypic diversity overall from 2009 and over seed increase from G_0 in 2010.

There were not significant differences between years or among populations for plant dry weight (Table 2-1). The mean plant dry weight for the G_0 individuals was 0.110 kg per individual (Table 2-2). The mean plant dry weight for the individuals of the six increase populations was 0.099 to 0.116 kg, or 90.0% to 105.5% of that of G_0. The standard error of the plant dry weight mean for the G_0 individuals was 0.001 kg (Table 2-3). The standard errors for the plant dry weight means for the individuals of the six seed increase populations were similar, 0.001 to 0.002 kg, indicating no change in phenotypic diversity over seed increase.

Leaf Type

Significant differences were found between years but not among populations for leaf type scores (Table 2-1). The mean leaf type score for the G_0 individuals was 3.3 in 2009 and 3.3 to 3.4 for the six increase populations (Table 2-2 and Figure 2-2). The mean leaf type score for the G_0 individuals grown in 2010 was 4.2, 0.9 higher than in 2009. The mean leaf type score for the individuals of the six seed increase populations was 3.8 to 4.2, similar to that of G_0. The standard errors for the mean leaf type scores for the individuals of the G_0 populations were 0.05 and 0.02 in 2009 and 2010, respectively, and were similar for the individuals of the six seed increase populations, 0.04 to 0.07 in 2009 and 0.01 to 0.03 in 2010 (Table 2-3), indicating no change in phenotypic diversity over seed increase.

Flower Characteristics

Days to flower (DTF)

Significant differences were found for DTF between years and among populations in 2010 but not in 2009 (Table 2-1). In 2009 the mean number of DTF for the G_0 individuals was 109.1 days (Table 2-2). The mean number of DTF for the six increase populations was 109.9 to 113.2 days, similar to that of G_0. The mean number of DTF for the G_0 individuals in 2010 was 7.1% smaller (101.3 days). The mean number of DTF for the individuals of the six increase populations in 2010 was 98.9 to 103.5 days, similar to the mean DTF of G_0. The significant difference detected among populations in 2010 was among G_1C, G_3C, G_1N and G_2N. The first three populations took fewer DTF (98.9 to 99.1 days), while the last population took significantly more DTF (103.5 days).

The standard error for the mean DTF was 0.84 days for the G_0 individuals in 2009 and 0.55 to 0.71 days for the individuals of the six increase populations (Table 2-3), indicating a slight decrease in phenotypic diversity over seed increase. In 2010 there was a decrease in the standard error for the mean DTF, which was 0.17 days for the G_0 individuals and 0.14 to 0.29 days for the individuals of the six increase populations, indicating an overall decrease in phenotypic diversity from 2009 but not over seed increase within 2010.

Disk flower size (DFS)

There were significant differences for DFS between years but not among populations (Table 2-1). The mean DFS for the G_0 individuals was 0.79 cm in 2009 (Table 2-2). The mean DFS for the individuals of the six increase populations ranged from 0.78 to 0.83 cm, similar to that of G_0. The mean DFS for the G_0 individuals grown

in 2010 was 0.88 cm, 11.4% larger than the mean size in 2009. DFS increased by 3.6% to 15.4% (0.86 to 0.90 cm) for all six increase populations. The mean DFS for the increase populations in 2010 was 97.7% to 102.3% of the mean DFS of the G_0 individuals, similar to the 2009 results.

The standard error for the mean DFS was 0.009 cm for the G_0 individuals in 2009 and 0.004 to 0.007 cm for the individuals of the six increase populations (Table 2-3), indicating no change in phenotypic diversity over seed increase. In 2010 there was a decrease in standard error, where the standard error for the mean DFS was 0.001 cm for the G_0 individuals and 0.002 to 0.007 cm for the individuals of the six increase populations, indicating an overall decrease in phenotypic diversity from 2009 but not over seed increase within 2010.

Whole flower size (WFS)

There were significant differences for WFS between years but not among populations (Table 2-1). The mean WFS was 3.3 cm for the G_0 individuals in 2009 (Table 2-2). Similar mean WFS (3.4 to 3.5 cm) were recorded for the individuals in the six increase populations. The mean WFS for the G_0 individuals in 2010 was 9.1% smaller (3.0 cm) than in 2009. A similar trend (11.8% to 17.1% flower size decrease) was observed for all six increase populations. Again, the mean WFS for the six increase populations in 2010 (2.9 to 3.0 cm) was similar to that of G_0.

The standard error for the mean WFS was 0.019 cm for the G_0 individuals in 2009 and 0.021 to 0.033 cm for the six increase populations (Table 2-3), indicating no change in phenotypic diversity over seed increase. In 2010 there was a decrease in standard error for the mean WFS for the G_0 individuals at 0.012 and was 0.005 to 0.011 cm for

the six seed increase populations, indicating an overall decrease in phenotypic diversity from 2009 but not over seed increase within 2010.

Petal lobing

No significant differences were found between years or among populations for petal lobing (Table 2-1). The mean petal lobing score was 4.2 for the G_0 individuals and 4.2 to 4.9 for the six increase populations (Table 2-2 and Figure 2-4). The standard error for the mean petal lobing score was 0.10 for the G_0 individuals and 0.01 to 0.10 for the individuals of the six increase populations (Table 2-3), indicating no change in phenotypic diversity over seed increase.

Degree of petal overlap (DPO)

There were significant differences for the DPO between years and among populations (Table 2-1). The average rating for the DPO was 2.6 for the G_0 individuals in 2009 (Table 2-2 and Figure 2-6). All three populations produced in central Florida had mean ratings of 2.7. The mean ratings for the populations produced in northern Florida were 2.2 (G_2N) to 2.4 (G_1N and G_3N), but were not significantly different. G_2N was significantly different from the populations produced in central Florida.

The ratings decreased in 2010 compared to 2009 for all populations (Table 2-2 and Figure 2-6). The mean rating for DPO in 2010 was 2.4 for the G_0 individuals, a 0.2 decrease from 2009. The mean rating for the populations produced in central Florida were from 2.3 to 2.4, a decrease of 0.3 to 0.4 from 2009. The mean ratings for the populations produced in northern Florida were from 2.0 to 2.2, a decrease of 0.2 to 0.3 from 2009. The mean rating for G_2N was significantly different from G_1C, G_2C and G_0.

The standard error for the mean DPO scores for the G_0 individuals was 0.037 and 0.030 to 0.065 for the individuals of the six seed increase populations in 2009 (Table 2-

3), indicating no change in phenotypic diversity over seed increase. Standard error for the mean DPO scores decreased in 2010, where the standard error was 0.017 for the G_0 individuals and ranged from 0.019 to 0.028 for the individuals of the six seed increase populations, indicating an overall decrease in phenotypic diversity from 2009 but not over seed increase within 2010.

Number of ray petals per flower head (NRP)

No significant differences were detected between years or among populations for the NRP per flower head (Table 2-1). The mean NRP per flower head was 8.12 for the G_0 individuals (Table 2-2). The mean NRP for the individuals of the six increase populations was 8.04 to 8.22. The standard error for the mean NRP for the G_0 individuals was 0.020 and 0.006 to 0.059 petals for the six increase populations (Table 2-3), indicating no change in phenotypic diversity over seed increase.

Seed Production and Germination

There were significant differences between years and among populations for both years for seed production for five seed heads per plant (Table 2-1). The mean amount of seed produced for the G_0 individuals was 590.0 seed in 2009 (Figure 2-7). The mean seed production for the three central Florida increase populations ranged from 540.9 to 652.4 seed, not significantly different from that of G_0 but G_2C (540.9 seed) was significantly different from G_3C (652.4 seed). G_1N (425.8 seed) produced significantly less seed than G_2N (570.5 seed) and G_3N (588.8 seed) as well as G_0 and the populations from central Florida.

Seed production of G_0 increased by 1.7% (599.9 seed) in 2010 compared to 2009 (Figure 2-7). Except for one increase population (G_3C), seed production of the increase populations increased by 17.4% to 46.3% reaching 623.0 to 737.9 seed in 2010. G_1C

(722.5 seed), G_2C (644.0 seed), G_3C (632.7 seed), G_1N (623.0 seed) and G_3N (691.3 seed) did not produce significantly more seed than G_0, but G_2N (757.9 seed) did.

Standard error for the mean number of seed produced for five seed heads was 10.31 seed for the G_0 individuals and 6.91 to 12.76 seed for the individuals in the six increase populations in 2009 (Table 2-3), indicating no change in phenotypic diversity over seed increase. Standard error for the mean number of seed produced for five seed heads for the G_0 individuals was lower in 2010, 3.27 seed for the G_0 individuals and 4.06 to 12.47 seed for the individuals of the six increase populations, indicating an overall decrease in phenotypic diversity from 2009 but not over seed increase within 2010.

There were not any significant differences found among populations for seed germination in 2009 (Table 2-1). Mean seed germination was 56.6% for the G_0 individuals and 52.1% to 61.2% for the individuals of the six increase populations (Table 2-2). The standard error was 1.34% for G_0 and 1.06% to 1.48% for the six increase populations (Table 2-3), indicating no change in phenotypic diversity over seed increase.

Powdery Mildew Severity (PMS)

Significant differences were found between years but not among populations for PMS (Table 2-1). The mean severity score for PMS for the G_0 individuals was 5.5 and 4.9 to 5.8 for the individuals in the six seed increase populations in 2009 (Table 2-2 and Figure 2-8). The mean PMS score decreased in 2010 for G_0 and the six increase populations compared to 2009. The mean PMS score was 3.0 for the G_0 individuals and 2.5 to 3.2 for the individuals of the six seed increase populations in 2010.

Standard error for the mean PMS ratings in 2009 for the G_0 individuals was 0.16 and 0.10 to 0.19 for the individuals of the six seed increase populations (Table 2-3), indicating no change in phenotypic diversity over seed increase. Standard error for the mean PMS ratings decreased slightly in 2010, which was 0.11 for the G_0 individuals and 0.05 to 0.10 for the individuals of the six seed increase populations, indicating no change in phenotypic diversity over seed increase.

Principal Component Analysis

A dataset of 168 data points was assembled from the mean values of seven populations for 12 characteristics in two years (7 x 12 x 2). Principal component analysis of this dataset revealed that two principal components could account for 78.2% of the total observed variance. The first principal component had an eigenvalue of 7.35 and could account for 61.2% of the total variance. The second principal component had an eigenvalue of 2.04 and could account for 17.0% of the total variance. The remaining components had eigenvalues ≤1.04 and failed to explain much variance, so they were excluded from further analysis. Six characteristics (DFS, DTF, leaf type, plant height, PMS and WFS) influenced the first principal component heavily, with loading values ≥0.93. The second principal component was most influenced by the NRP, plant dry weight and seed germination, with loading values ≥0.64. Based on these two principal components, the populations were clustered primarily by year (Figure 2-9). Within the 2009 cluster, the northern and the central Florida populations appeared to be separated. However, this clustering did not show up in 2010. The lack of consistent, clear clustering among the populations from year to year suggests that no obvious population differentiation had occurred during the three successive generations of seed increase in either location, based on the phenotypic characteristics evaluated.

Discussion

The seed germination tests in the current study did not take dormant seed into account by performing seed viability tests of non-germinated seed. Seed of *C. leavenworthii* has been reported as exhibiting after-ripening and the viability declined with longer storage (Norcini and Aldrich, 2007). Kabat et al. (2007) reported that a small percentage of fresh seed were nondormant and most were viable, dormant seed. Most of the seed gradually became nondormant, and a high percentage of viable seed would germinate when exposed to warm, moist conditions under a wide range of temperatures commonly found in Florida. Based on germination and tetrazolium staining tests, seed viability of *C. leavenworthii* seeds has been estimated at 57.0% (Norcini and Aldrich, 2008). The time of year of seed production and collection can affect seed quality, where seed that were harvested from May through July were of the best quality (Norcini et al., 2006). Seed viability was about 75% for this time period with fertilizer enhancing seed production.

Unintentional selection was minimized by transplanting the first 128 randomly selected seedlings to produce the next generation of seed increase. The most vigorous or first germinated seedlings were not transplanted over weaker seedlings. The only type of unintentional selection that could have occurred was the exclusion of seedlings from dormant seed. Dormant seed need specific environmental conditions to germinate that were not used. This could have led to fewer seedlings in later generations that did not exhibit seed dormancy.

Although *C. leavenworthii* has been documented as an outcrossing species, it can still have effects from inbreeding depression through biparental inbreeding, as reported in *Gaillardia pulchella* (Heywood, 1993). Biparental inbreeding can be caused by

outcrossing between genetic relatives, where there can be a decline in fitness in progeny of related individuals than nonrelated individuals. This can also cause a reduction in seed production in species that exhibit self incompatibility, where *C. leavenworthii* has been reported as sporophytically self incompatible.

The increase populations had similar mean values with G_0 over both years for nine out of the 12 characteristics evaluated, including four characteristics (leaf type, petal lobing, DPO and PMS) evaluated on rating scales. There were significant differences among populations for DTF in 2010 but not in 2009, DPO over both years and seed production over both years. These differences were not consistent by generation or location, indicating that no genetic shift occurred by the seed increase practices.

In PCA, the northern Florida populations appeared to be clustered away from the central Florida populations in 2009, but that clustering disappeared in 2010. There were not consistent changes in phenotypic diversity to indicate that location had an effect on seed increase over successive generations. Considering these results, it can be concluded that three generations of successive seed increase in central or northern Florida will not likely to cause significant population differentiation or genetic shift in *C. leavenworthii* seed production on plant growth and development, leaf morphology, flowering and flower morphology, seed production and powdery mildew resistance.

Simon and Kastenbauer (1979) found similar results when meadow fescue, timothy and perennial ryegrass were multiplied over two generations. These forage species were relatively unaffected by multiplication over two generations. Rincker et al. (1982) found no consistent shifts in plant growth characteristics evaluated for Italian ryegrass in two and three generations evaluated.

There were yearly effects found for the seed increase populations at both locations, possibly due to fertilization, irrigation, humidity, day length and differences in the time of year of the growing seasons. It is difficult to predict which factors affected phenotypic changes between years and because there were not any consistent changes in populations over successive generations, these factors did not seem to have a detrimental effect on phenotypic diversity. Each year will be different when producing seed over successive generations and multiple locations. This illustrates the importance of using a phenotypically and genetically diverse starting population and maintaining it in each successive generation to have the ability to adjust to changing environmental conditions. When the population by year interaction (genotype x environment) was tested, there was only one significant interaction found, which was for seed production. Because the years were significantly different and most of the population by year interactions were not significant, the years were treated separately. This indicated that the environmental conditions had an effect on the phenotype of most of the traits evaluated, but the effects were consistent within each year.

Summary

After evaluating these populations over two years, there were no clear trends of genetic shift in *C. leavenworthii* using the current commercial seed production practices due to generation or location. The standard error values indicated no change in variation of the traits evaluated. It is apparent that environmental conditions can cause changes in some of the phenotypic characteristics of this species but did not affect the phenotypic diversity in the current study. Even though there were differences between years, the results within each year did not change in generation or location due to seed increase practices.

Table 2-1. Analysis of variance of several phenotypic characteristics for seven *C. leavenworthii* populations produced in two locations and grown at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010.

		Between Years		Years*Populations		Among Populations[z]	
Plant Characteristic	Year	F value	Probability	F value	Probability	F value	Probability
Plant height	2009	95.26	0.0001	0.43	0.8590	0.98	0.4422
	2010					1.93	0.1162
Plant dry weight	Combined	0.00	0.9531	1.27	0.2782	1.38	0.2265
Leaf type	2009	34.06	0.0001	0.64	0.6951	0.44	0.8473
	2010					2.60	0.0439
Days to flower	2009	96.35	0.0001	0.76	0.6060	1.18	0.3236
	2010					4.02	0.0063
Disk flower diameter	2009	19.04	0.0001	0.96	0.4590	1.73	0.1240
	2010					1.22	0.3299
Whole flower diameter	2009	162.93	0.0001	0.56	0.7591	1.25	0.2876
	2010					1.89	0.1233
Petal lobing	Combined	6.44	0.9861	0.61	0.7226	1.81	0.1028
Petal overlap	2009	0.00	0.0125	0.25	0.9585	4.33	0.0008
	2010					3.98	0.0067
Ray petals (No.)	Combined	0.98	0.3254	0.30	0.9365	0.97	0.4463
Seed production	2009	23.53	0.0001	3.08	0.0079	8.50	0.0001
	2010					3.21	0.0186
Seed germination	2009	NA	NA	NA	NA	1.44	0.2099
Powdery mildew severity	2009	70.7	0.0001	0.48	0.825	0.99	0.4353
	2010					1.63	0.1832

[z]Sample size among blocks was 5 in 2009 and 15 in 2010 and 75 among populations for both years.

Table 2-2. Comparison of several phenotypic characteristics of seven *C. leavenworthii* populations produced at two locations and grown at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010.

Plant Characteristic	Year	G_0	G_1C	G_2C	G_3C	G_1N	G_2N	G_3N
Plant height (cm)	2009	72.3[ns]	73.1	73.0	73.8	69.1	71.8	73.9
	2010	56.7[ns]	58.9	56.4	55.1	56.6	57.2	61.0
Plant dry weight (kg)	Combined	0.110[ns]	0.116	0.114	0.114	0.103	0.099	0.105
Leaf type	2009	3.3[ns]	3.4	3.4	3.4	3.3	3.3	3.3
	2010	4.2[ns]	4.1	4.0	3.9	3.8	4.2	4.0
Days to flower (days)	2009	109.1[ns]	112.2	112.3	112.6	109.9	112.2	113.2
	2010	101.3ab[z]	99.1b	100.2ab	98.9b	99.0b	103.5a	100.8ab
Disk flower size (cm)	2009	0.79[ns]	0.83	0.78	0.78	0.79	0.82	0.80
	2010	0.88[ns]	0.86	0.87	0.90	0.88	0.90	0.88
Whole flower size (cm)	2009	3.3[ns]	3.4	3.5	3.4	3.4	3.5	3.5
	2010	3.0[ns]	2.9	2.9	3.0	2.9	3.0	3.0
Petal lobing	Combined	4.2[ns]	4.6	4.4	4.4	4.2	4.9	4.5
Petal overlap	2009	2.6ab[z]	2.7a	2.7a	2.7a	2.4ab	2.2b	2.4ab
	2010	2.4a[z]	2.4a	2.4a	2.3ab	2.2ab	2.0b	2.1ab
Ray petals (no.)	Combined	8.12[ns]	8.05	8.08	8.04	8.22	8.07	8.10
Seed germination (%)	2009	56.6[ns]	52.1	61.2	60.2	58.3	55.6	59.7
Powdery mildew severity	2009	5.5[ns]	4.9	5.3	5.5	5.3	5.4	5.8
	2010	3.0[ns]	3.1	3.0	3.0	3.0	2.5	3.2

[ns]Means in rows not found to be significantly different by Tukey's W Procedure at p≤0.05.
[z]Significant differences in rows determined by Tukey's W Procedure at p≤0.05.

Table 2-3. Standard errors of several phenotypic characteristics of seven *C. leavenworthii* populations produced at two locations and grown at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010.

Plant Characteristic	Year	G_0	G_1C	G_2C	G_3C	G_1N	G_2N	G_3N
Plant height (cm)	2009	0.77	0.95	0.72	0.75	0.75	0.90	0.70
	2010	0.73	0.41	0.61	0.46	0.50	0.66	0.42
Plant dry weight (kg)	Combined	0.001	0.002	0.002	0.002	0.002	0.002	0.001
Leaf type	2009	0.05	0.05	0.05	0.06	0.07	0.04	0.04
	2010	0.02	0.03	0.02	0.03	0.03	0.01	0.01
Days to flower (no.)	2009	0.84	0.58	0.71	0.55	0.69	0.70	0.61
	2010	0.17	0.27	0.14	0.29	0.16	0.23	0.20
Disk flower size (cm)	2009	0.009	0.004	0.007	0.007	0.006	0.005	0.005
	2010	0.001	0.003	0.004	0.007	0.003	0.004	0.002
Whole flower size (cm)	2009	0.019	0.021	0.030	0.025	0.029	0.033	0.028
	2010	0.012	0.006	0.009	0.011	0.010	0.005	0.010
Petal lobing	Combined	0.10	0.07	0.10	0.07	0.01	0.06	0.08
Petal overlap	2009	0.037	0.032	0.031	0.030	0.065	0.047	0.050
	2010	0.017	0.020	0.028	0.024	0.022	0.019	0.020
Ray petals (no.)	Combined	0.020	0.006	0.011	0.006	0.059	0.013	0.012
Seed production (no.)	2009	10.31	7.73	12.76	11.35	11.38	12.46	6.91
	2010	3.27	5.61	11.84	4.06	9.78	12.47	4.94
Seed germination (%)	2009	1.34	1.06	1.21	1.21	1.48	1.35	1.41
Powdery mildew severity	2009	0.16	0.14	0.17	0.19	0.12	0.17	0.10
	2010	0.11	0.07	0.10	0.08	0.07	0.08	0.05

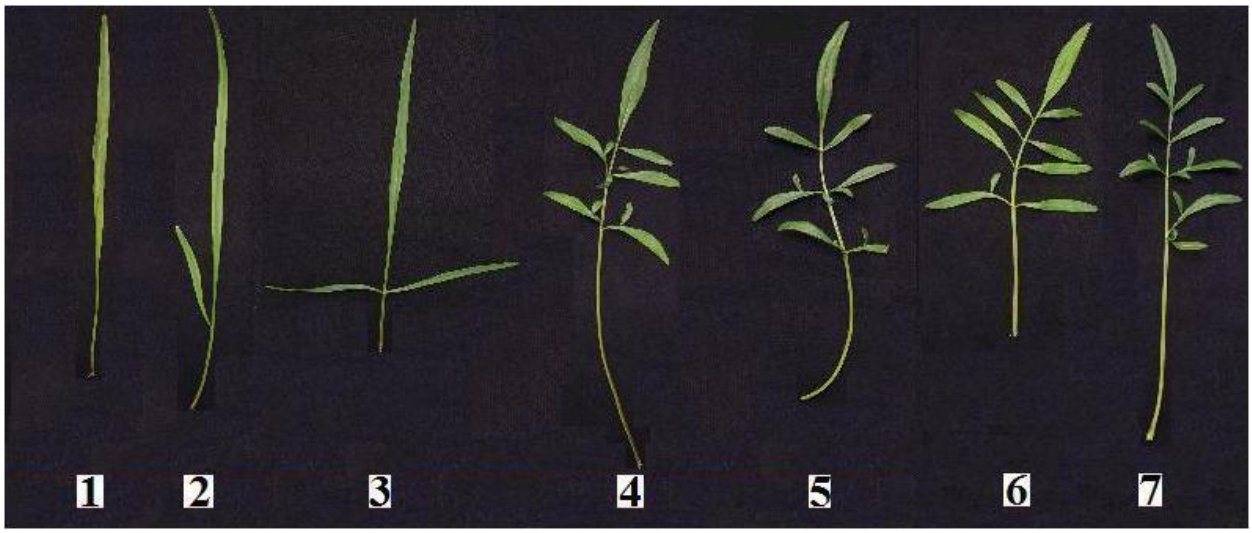

Figure 2-1. Leaf types observed in *C. leavenworthii* with assigned scores (Czarnecki et al., 2007).

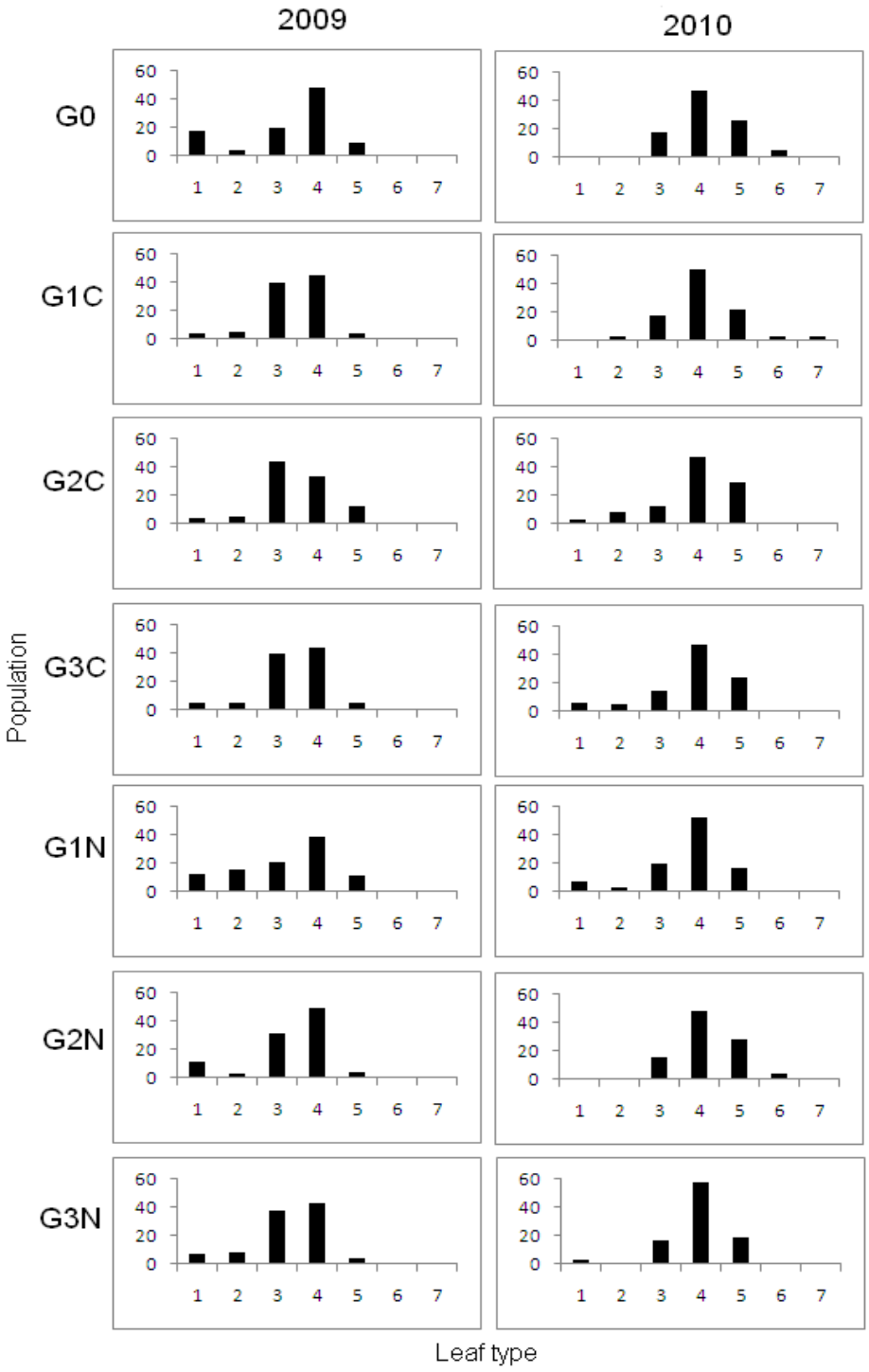

Figure 2-2. Distribution of leaf type scores in the natural population and six seed increase populations of *C. leavenworthii* produced at two locations and grown at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010. The Y-axis indicates the frequency of each score occurring for each generation at each location.

Figure 2-3. Scores used for evaluating petal lobing of seven *C. leavenworthii* populations produced at two locations and grown at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010.

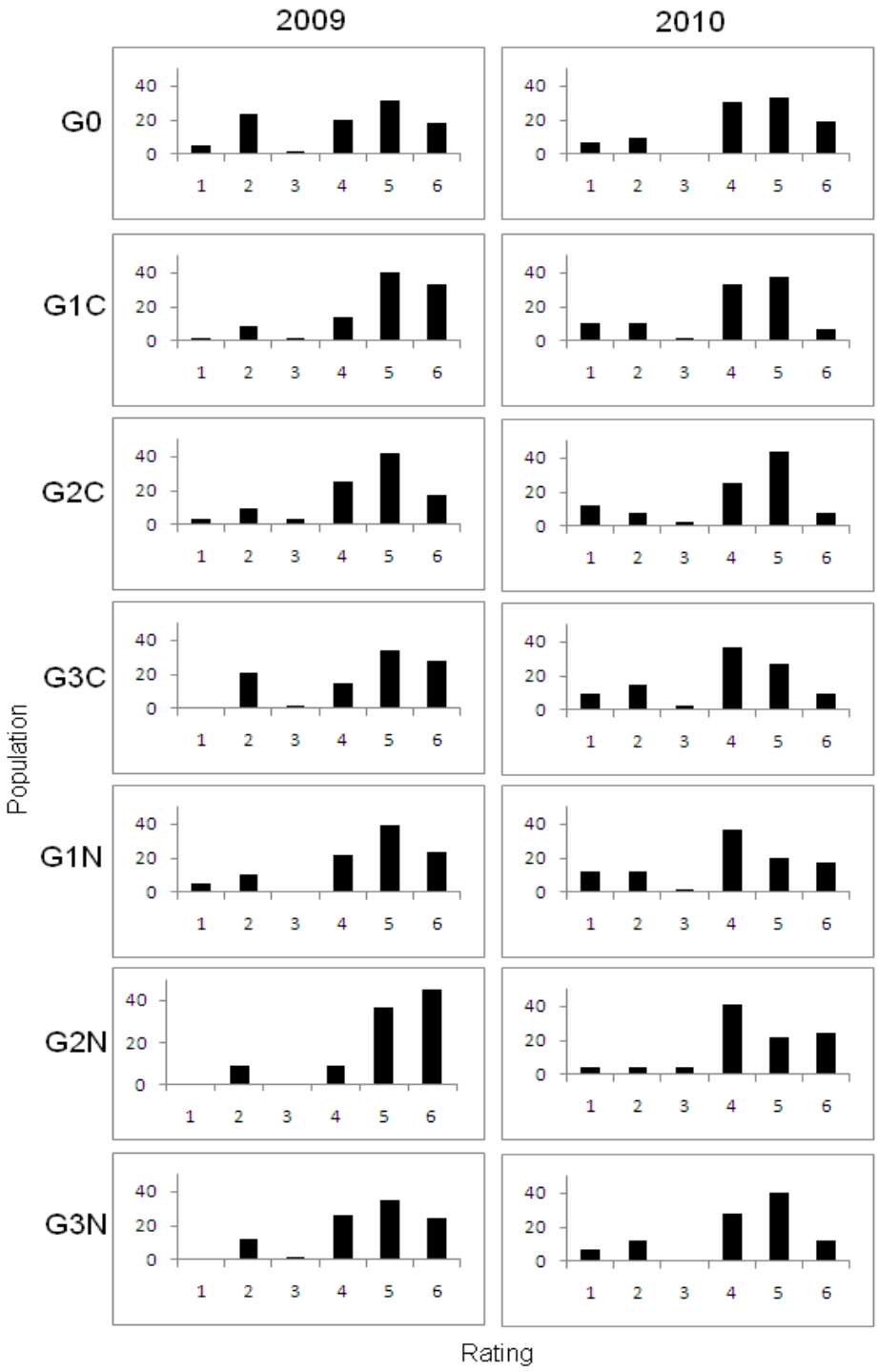

Figure 2-4. Changes in petal lobing scores for seven populations of *C. leavenworthii* produced at two locations and grown at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010. The Y-axis indicates the frequency of each score occurring for each generation at each location.

Figure 2-5. Scores used for evaluating petal orientations of seven *C. leavenworthii* populations produced at two locations and grown at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010. (G=1, S=2, O=3)

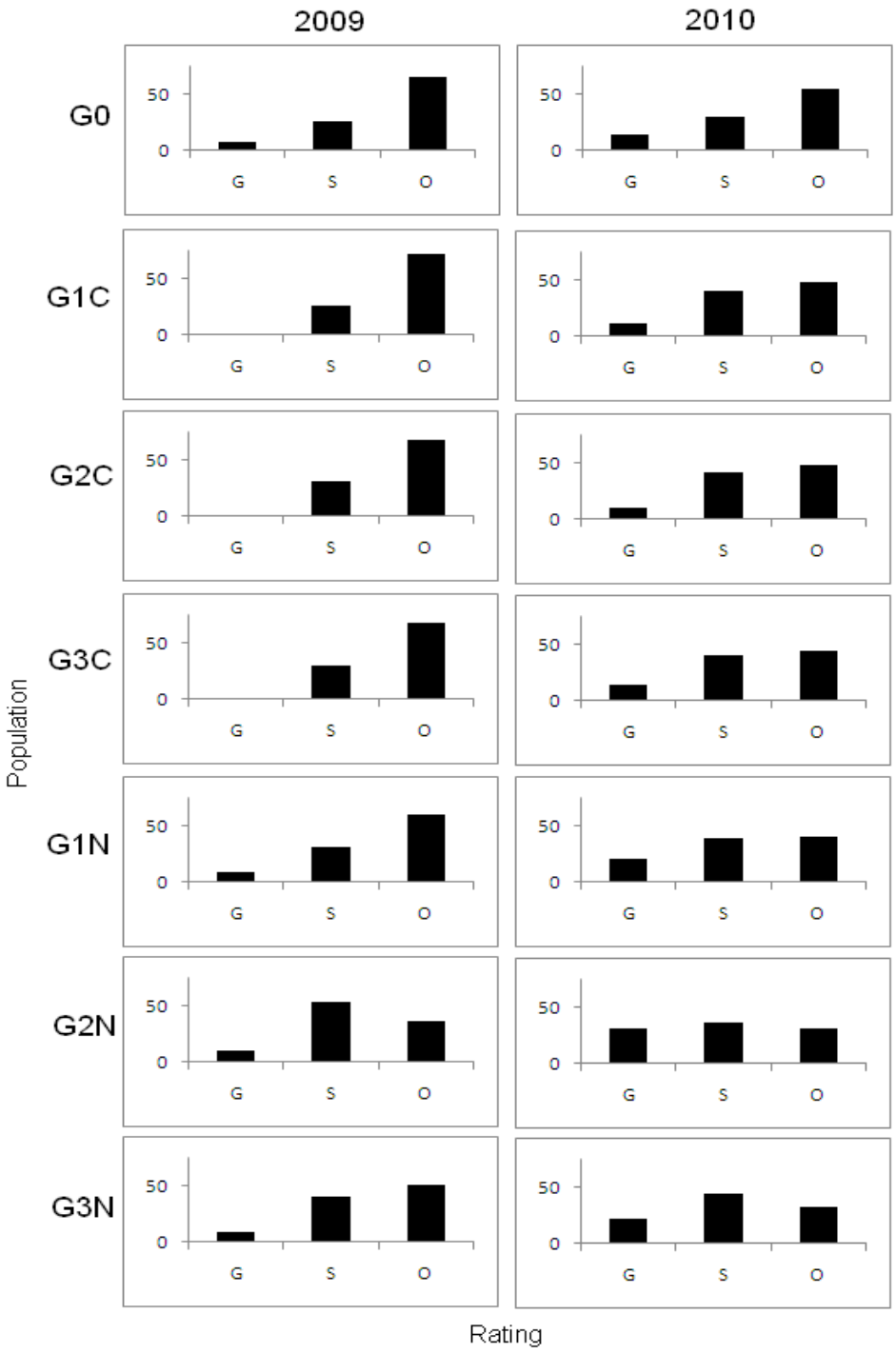

Figure 2-6. Changes in the degree of petal overlap ratings for seven populations of *C. leavenworthii* produced at two locations and grown at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010. The Y-axis indicates the frequency of each rating occurring for each generation at each location.

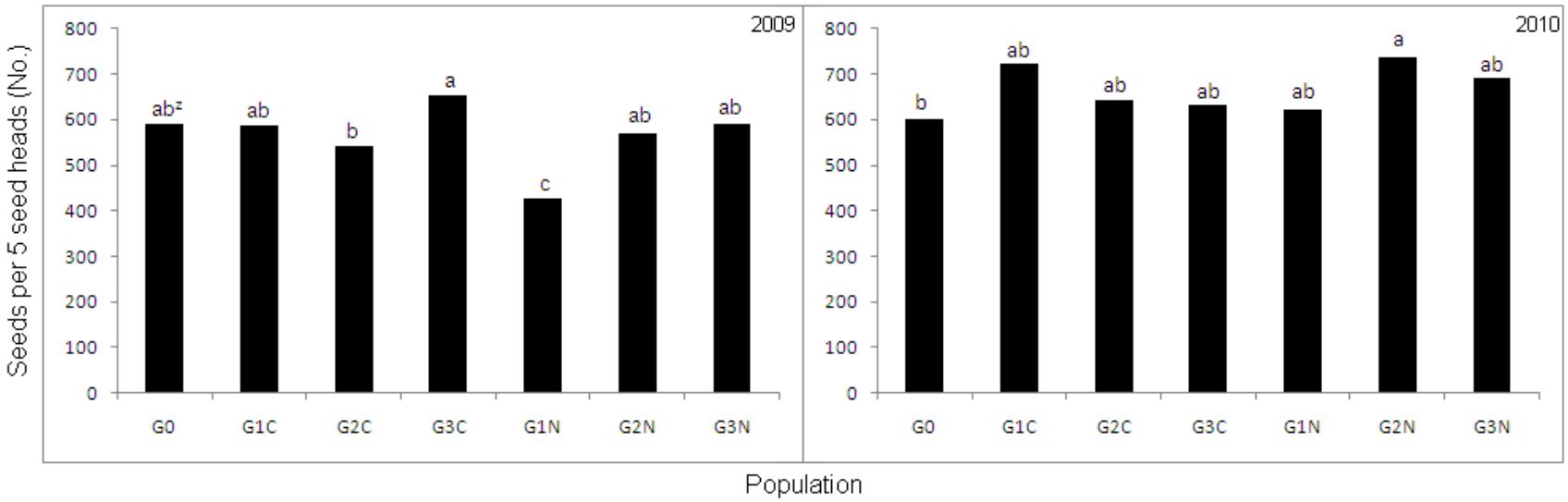

Figure 2-7. Differences in the number of seeds per five seed heads of seven *C. leavenworthii* populations produced at two locations and grown at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010. [z]Means within cultivar not followed by the same letter are significantly different by Tukey's W Procedure at p≤0.05 within each year. Means for each year were statistically analyzed separately and cannot be compared to each other.

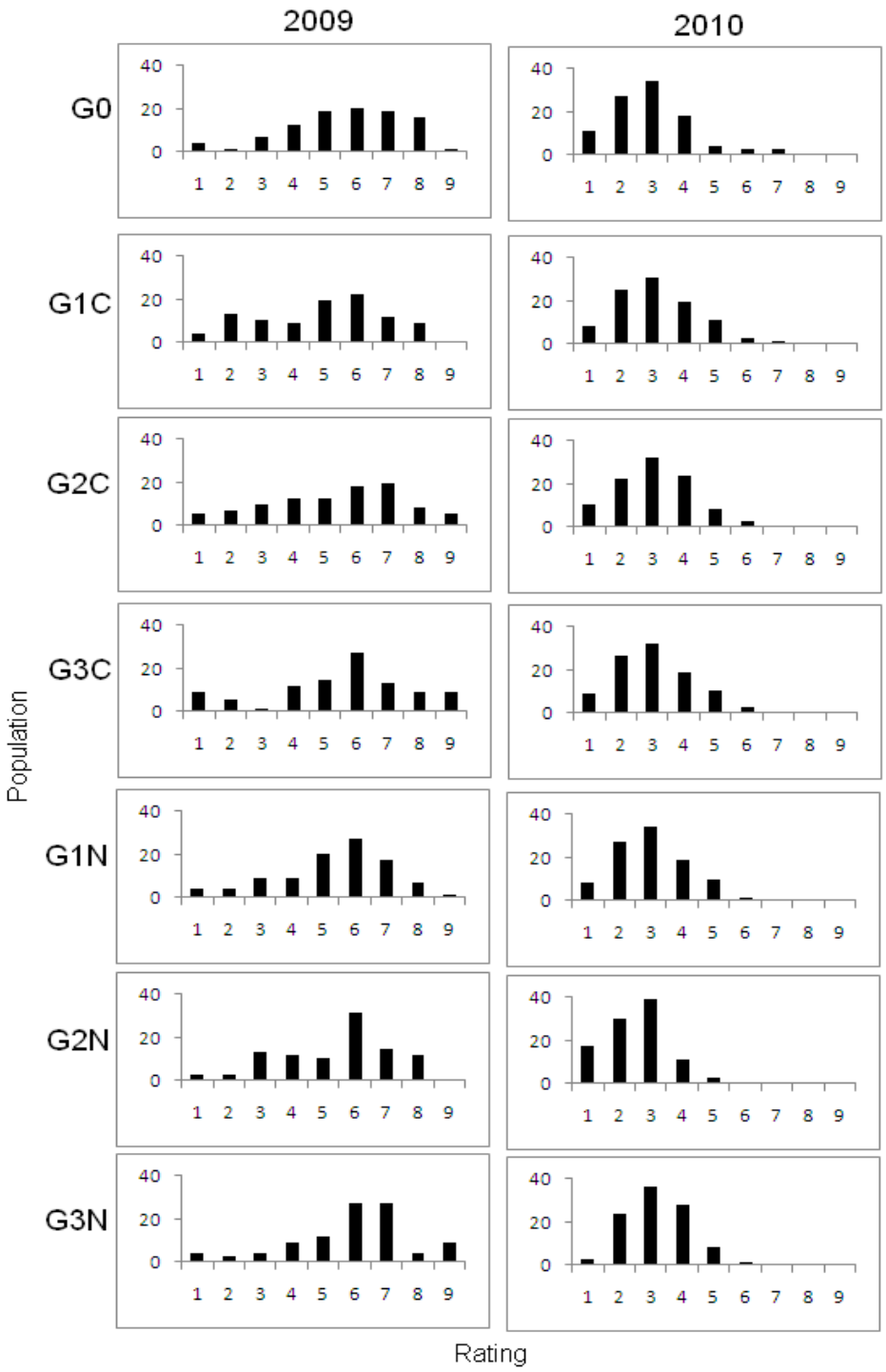

Figure 2-8. Distribution of powdery mildew severity among individuals for each of the seven populations of *C. leavenworthii* produced at two locations and grown at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010. The Y-axis indicates the frequency of each severity score occurring for each generation at each location.

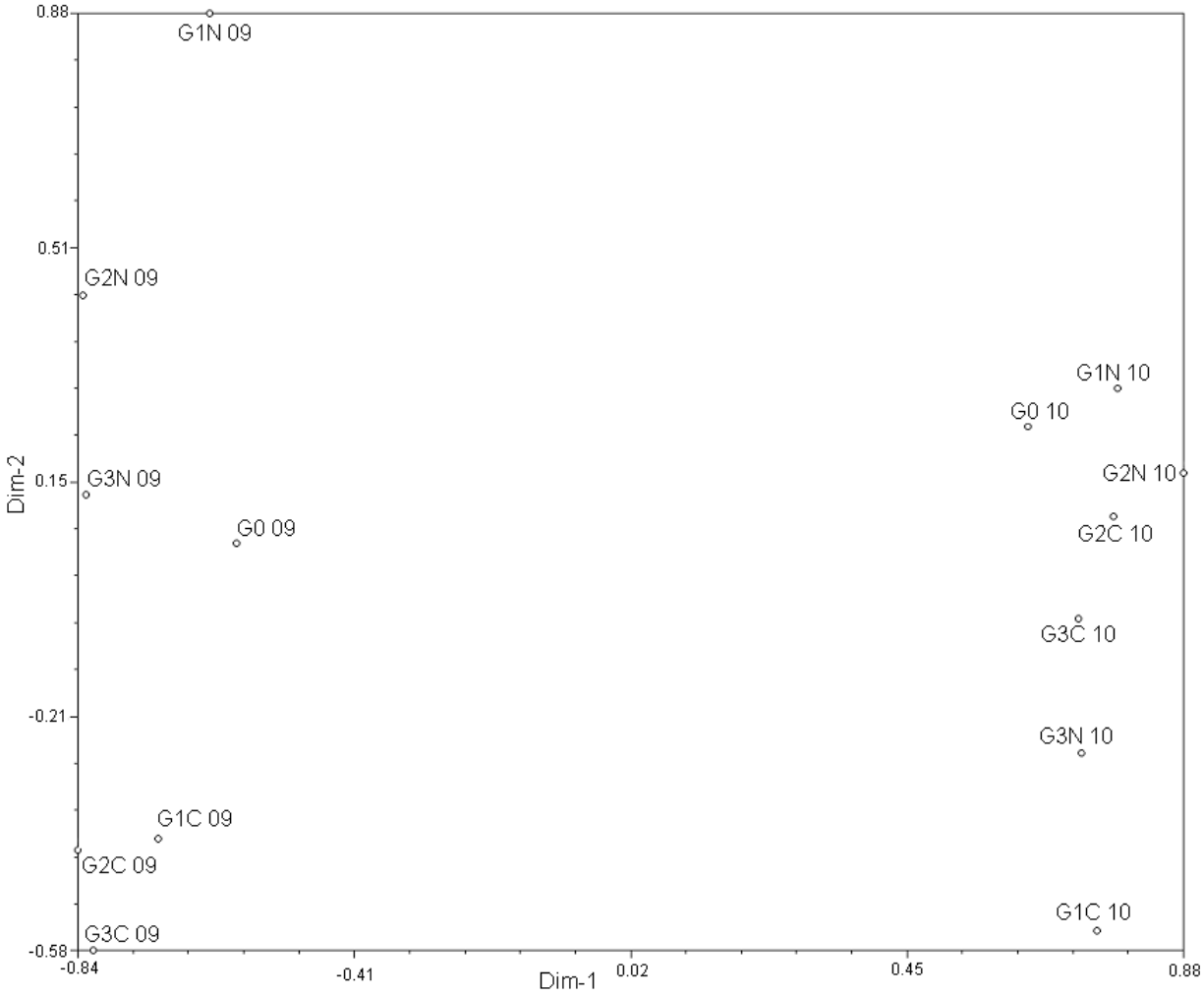

Figure 2-9. Principal component analysis of *C. leavenworthii* populations grown in 2009 and 2010 at the Gulf Coast Research and Education Center, Wimauma, FL.

CHAPTER 3
ASSESSING MOLECULAR CHANGES USING SIMPLE SEQUENCE REPEAT (SSR) MARKERS OF A NATURAL *COREOPSIS LEAVENWORTHII* POPULATION DURING SEED INCREASE

Justification

Coreopsis leavenworthii is endemic to Florida, solely produced in Florida and commonly planted along roadsides in highway beautification projects (Kabat et al., 2007; Norcini and Aldrich, 2007). Current *C. leavenworthii* commercial seed production practices start with a natural population and use a segment of that population to produce seed over numerous generations. Seed produced are required to not only be of high quality but also preserve the level of composition of genetic diversity of the original, naturally-occurring population (Hartl, 2000). Production practices, fertilization, soil moisture and soil type, can change and/or cause loss of genetic diversity in the produced seeds and affect plant performance and survival. For endemic species, these negative effects could be dramatic and impact the survival of the species.

In a previous study (Chapter 2) the effects of seed increase on the genetic diversity and integrity of *C. leavenworthii* populations were assessed based on phenotypic characterization. The information gained was very valuable, but the number of characteristics available for analysis was limited. In addition, the assessed characteristics were mostly quantitative, which were known to be prone to influences by many factors, such as growth conditions and plant developmental stages. Consequently subtle genetic changes (or genetic shifts) might have been masked by environmental effects and not identified. Therefore, there has been a strong need for the use of more powerful tools to assess and monitor potential genetic shifts during seed increase based on the practices used by *C. leavenworthii* seed producers, where

seed is collected from a natural population and increased for several generations. Molecular markers, especially those based on DNA polymorphisms, can be desirable tools for such assessment and monitoring because they cannot be affected by environmental changes, plant developmental stages or physiological status, and can be analyzed early and reliably.

A number of molecular marker systems have been used to assess the genetic diversity and composition of plant populations or species, including allozymes, RAPDs, RFLPs, AFLPs and SSRs. Among them AFLP and SSR markers have been the most commonly used systems for genetic diversity and relationship analysis (Mueller and Wolfenbarger, 1999; Weising et al., 2005). For AFLP analysis, prior knowledge of the DNA sequence has not been required, a great number of markers have been produced and they have been reported as reliable, reproducible, affordable and fast to generate (Blears et al., 1998; Mueller and Wolfenbarger, 1999; Vos et al., 1995). AFLP markers have been reported as dominant and the DNA must be of high purity for complete digestion by the restriction enzymes. On the other hand, SSR markers have been reported as codominant, detected a high level of polymorphism in genetic diversity studies and were widely distributed in higher plants (Blears et al., 1998; Mueller and Wolfenbarger, 1999). SSR markers were species-specific, thus they typically must be designed for species to be analyzed, which has been time consuming and costly (Glenn and Schable, 2005; Goldstein and Pollock, 1997; Zane et al., 2002).

For example, AFLP markers have been used to assess genetic changes in awned slender wheatgrass over three generations (Ferdinandez et al., 2005). The authors revealed a significant reduction in marker variation in the first and second generations

and a large portion of AFLP markers changed frequencies. The change in band frequency indicated a genetic shift in two generations of seed increase with the possibility of further losses in later generations. In another study AFLP markers were used to assess the genetic diversity of a composite of four blue grama seed sources over three generations (Fu et al., 2004). The AFLP markers detected some genetic change over three generations but not enough to indicate a genetic shift. The composite of seed was able to maintain a high level of genetic diversity over three generations of seed production. SSR markers were used to determine if the genetic integrity of wheat was being upheld for up to 24 multiplications (Borner et al., 2000). There was no contamination found and a high level of genetic identity was upheld in the wheat multiplication populations. The genetic identity of rye was assessed using SSR markers over 7-13 generations of multiplication, where there was significantly different allele frequencies detected in four out of six accessions (Chebotar et al., 2003). These studies illustrated the usefulness of molecular markers in evaluating the genetic diversity and integrity over generations of seed increase.

Czarnecki et al. (2008) detected a high level of total genetic diversity (H_T=0.309) in natural *C. leavenworthii* populations from north, central and south Florida using AFLP markers. Their studies also showed that seed production populations that originated in central Florida and were increased in northern and central Florida for a various number of generations (G_1-G_4) maintained a relatively high level of genetic diversity (0.251). It was not known if producing seed from a starting natural population for several generations or in different locations, especially those produced in different locations than the origin of the natural population, affects the genetic diversity of *C. leavenworthii*.

Although the phenotypic work on these populations over two years (Chapter 2) indicated that the phenotypic diversity was not affected by generation or location, a molecular study was needed to compliment these findings to determine if genetic changes or shifts occurred over several generations of seed increase at two locations. The objectives of the current study were to use SSR markers to assess potential genetic changes that might occur to *C. leavenworthii* populations during seed increase in central and northern Florida over three generations to determine if an acceptable level of genetic diversity and integrity was maintained over seed increase and, if changes were occurring, quantify the nature and rate of changes.

Materials and Methods

Plant Populations

Seed collected from a *C. leavenworthii* natural population in Reedy Creek Mitigation Bank in Polk County, FL (Lat. 27°51' N, Long. -81°41' W, USDA cold hardiness zone 9a, AHS heat zone 11) was used as the seed source for subsequent increases. This source was referred to as Generation 0 (G_0). The collection consisted of 1220 seed heads from 122 individuals randomly selected out of the natural population (10 seed heads per individual). One seed lot from the natural population was sent to the North Florida Research and Education Center (NFREC), Quincy, FL (Lat. 30°32' N, Long. -84°35' W, USDA cold hardiness zone 8b, AHS heat zone 9) in northern Florida and another was kept at the Gulf Coast Research and Education Center (GCREC), Wimauma, FL (Lat. 27°45' N, Long. -82°13' W, USDA cold hardiness zone 9b, AHS heat zone 10) in central Florida. At each location, seed was increased successively for three generations (G_1, G_2 and G_3), starting in spring 2007, by growing ~100 plants from seed of a previous generation and harvesting mature seed heads from

ten open-pollinated flowers from each plant. Harvested seed heads were dried indoors at each site and then stored at the GCREC's seed storage room (10°C and 50% RH). The seed increase population was designated by its generation and its site of increase (C for the central Florida site and N for the northern Florida site). Therefore, the generations produced in central Florida were labeled G_1C, G_2C and G_3C and those produced in northern Florida were labeled G_1N, G_2N and G_3N. Additional information on the production of these populations can be found in Chapter 2.

Plant Tissue Collection and DNA Extraction

Approximately 50-100 mg of young leaf tissue was collected from 55 individuals of each population and dried in a dark container with silica gel beads for about three days. DNA was extracted using the microprep protocol of Fulton et al. (1995) and dissolved in 1 x TE buffer (pH 8.0, 10 mM Tris·Cl and 1 mM EDTA). DNA concentrations were determined using the Nanodrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE) and adjusted to reach an 8-10 ng/µL concentration.

SSR Marker Analysis

Polymerase chain reactions (PCRs) were carried out on an Eppendorf Vapo.protect Mastercycler Pro 384 (Eppendorf, Westbury, NY) (Table 3-1). Each reaction was done in a 10-µL volume containing 1 x PCR buffer (New England Biolabs, Ipswich, MA, USA), 1.5 mM of $MgCl_2$, 2 mM dNTPs, 0.25 pmol of forward primer with an M13 tail added to its 5' end, 2.5 pmol of reverse primer, 2.25 pmol of IRD700 or IRD800 infared dye-labeled M13 tail primer (Eurofins MWG Operon, Huntsville, AL, USA), and 0.25 units of Taq DNA polymerase with 8 ng of genomic DNA. The forward and reverse primers for each SSR marker were designed from *Coreopsis* genomic DNA sequences enriched with the GA motif (L. Gong and Z. Deng, unpublished). Depending on the

SSR markers, one of two thermal cycling programs was used for performing PCRs. The first cycling program started with initial denaturation at 95°C for 45 s followed by 10 cycles of 95°C for 45 s, 55°C for 45 s and 72°C for 60 s followed by 30 cycles of 95°C for 45 s, 45°C for 45 s, and 72°C for 60 s with a final extension of 72°C for 5 min. The second cycling program started with initial denaturation at 95°C for 45 s followed by 7 cycles of 95°C for 45 s, 68°C for 45 s and 72°C for 60 s followed by 31 cycles of 95°C for 45 s, 50°C for 45 s and 72°C for 30 s with a final extension of 72°C for 5 min. PCR-amplified DNA fragments were denatured at 95°C for 5 min and then separated in 6.5% denaturing polyacrylamide gels (18 cm long, 1 x TBE buffer) on a LI-COR 4300 DNA Analyzer (LI-COR biosciences, Lincoln, NE, USA). The DNA Analyzer was pre-run for 25 min at 1500 V, 40mA current, and 45°C. After the pre-run cycle, about 0.8-µL PCR reaction was loaded to each well, and the DNA Analyzer was run for about 1.5 h at 1500 V, 40 mA and 45°C. After the DNA samples were finished running on the gel, a picture of the gel was stored for later scoring. The bands were scored manually in an absent (0) or present (1) fashion (Figure 3-1).

Data Analysis

Population genetics computer programs were used to calculate the genetic diversity within populations, the genetic differentiation among populations and genetic distances among populations. Total genetic diversity (H_T) within and among populations was estimated, and the proportion of total genetic diversity residing among populations (G_{ST}) was calculated in POPGENE (Yeh et al., 1997), where $G_{ST} = (H_T - H_S)/H_T$ (Nei, 1987). The genetic relationships among populations were assessed based on the allele frequencies within populations and the pairwise genetic distances among populations that were calculated in POPGENE's Nei's unbiased algorithm (1978). The

G_{ST} values and genetic distances calculated were compared to the difference in generations between populations using PROC REG in SAS (SAS, 1997). The output from Nei's genetic distance matrices was then used in the MEGA program (Kumar et al., 2004) to generate an unweighted pair grouping method with arithmetic means (UPGMA) dendrogram of populations. The genetic relationships among populations were also assessed based on the SSR phenotypes and pairwise Apostol (or simple match) genetic distances. From the Apostol genetic distance matrix, a principal coordinate analysis (PCoA) was performed using DCENTER and EIGEN values provided in NYSYSpc (Rohlf, 2005). The genetic relationships among individuals were displayed in a two-dimensional plot using MXPLOT in NTSYSpc.

Results

SSR Alleles and Allele Frequencies

The 10 SSR primer pairs amplified a total of 104 alleles in 50 G_0 individuals and the number of alleles amplified per primer pair ranged from 4 to 17 with an average of 10.4 (Table 3-2), indicating a high level of genetic polymorphism at these SSR loci in the population. These primer pairs detected 97, 91 and 87 alleles in the G_1C, G_2C and G_3C individuals, respectively, and 97, 94 and 95 alleles in the G_1N, G_2N and G_3N individuals, respectively, although a similar number (46 to 52) of individuals was surveyed in each of the above populations. Compared to the G_0 population, the seed increase populations had 6.7% to 16.3% fewer alleles.

The reduction in allele number appeared to be more evident with certain SSR markers (e.g. COR7, COR10 and COR12) and in advanced generations (e.g. G_3C and G_3N) and COR4 in G_3C (Table 3-2). In some occasions (population G_3C and marker COR12), up to 35% of the G_0's alleles were not detected in the sampled individuals.

Marker COR4 detected 30% fewer alleles in both G_2C and G_3C, but did not show such a loss in G_1N to G_3N. Except for this marker, no obvious differences were observed between the two seed increase sites in allele loss. The number of alleles for the remaining six markers was relatively stable from G_0 through G_3 and between the two seed increase sites.

There were four alleles (COR18-1, COR8-5, COR4-11 and COR12-16) that were not detected in G_0 but appeared in one or more of the increased populations. There were two alleles that were present in G_0 but not observed in any of the increased populations. The lost alleles (COR7-13 and COR7-14) were both amplified by the same SSR marker, COR7.

The allele frequency in the G_0 population ranged from 0.010 to 0.853 per allele with an average of 0.151. A similar range of allele frequency (0.010 to 0.860) was observed in each of the increase populations. The average allele frequency in the increase populations fluctuated slightly, between 0.155 and 0.131, or between 102.7% and 86.8% of that of G_0.

To further assess potential changes in allele frequency, SSR alleles were separated into 11 groups based on their observed frequencies: (1) <0.05, (2) ≥0.05 to <0.15, (3) ≥0.15 to <0.25, (4) ≥ 0.25 to <0.35, (5) ≥0.35 to <0.45, (6) ≥0.45 to <0.55, (7) ≥0.55 to <0.65, (8) ≥0.65 to <0.75, (9) ≥0.75 to <0.85, (10) ≥0.85 to <0.95, and (11) ≥0.95, and the distribution of alleles in these categories were compared among populations (Figure 3-2). The most obvious changes appeared to be in Groups 1, 2 and 3. The G_0 population had 35 alleles in Group 1, 42 alleles in Group 2 and 15 alleles in Group 3 (Figure 3-2). The G_1C population had a similar number of alleles in Group 1

(35) and Group 2 (43), but fewer alleles in Group 3 (11). In G_2C and G_3C, more alleles fell into Group 1 (48-49), but fewer alleles in Group 2 (34-25) and Group 3 (8-12). A similar trend of change was evident in G_1N through G_3N, where more alleles had their frequencies <0.05 and fewer alleles had their frequencies between 0.05 and 0.25.

Total Genetic Diversity (H_T) within Populations

The H_T for the G_0 population was 0.1736 (Table 3-3). The H_T for the G_1C, G_2C and G_3C populations was 0.1666, 0.1558 and 0.1593, respectively, corresponding to 96.0%, 89.7% and 91.8% of the G_0 population's H_T. The H_T for the G_1N, G_2N and G_3N populations was 0.1706, 0.1590 and 0.1541, respectively, equivalent to 98.3%, 91.6% and 88.8% of that of the G_0 population. Thus, these data indicate 1.7% to 11.2% decrease in H_T within each increase population. The decrease appeared to be stabilized at around 90% of G_0's H_T. The change was similar between the two seed increase sites, or the decrease in H_T is independent of seed increase location.

Genetic Differentiation (G_{ST}) and Distances among Populations

G_{ST} values were calculated to determine the proportion of genetic diversity residing among populations. The remaining proportion of genetic diversity resides within populations (Berg and Hamrick, 1997). The G_{ST} value between G_0 and G_1C, G_2C or G_3C was 0.0244, 0.0394 and 0.0513, respectively, indicating a slight increase in G_{ST} or population differentiation with each successive generation (Table 3-3). The G_{ST} value between G_0 and G_1N, G_2N or G_3N was 0.0238, 0.0282 and 0.0399, respectively, indicating a similar increase in the G_{ST} value and population differentiation between the two seed increase sites. G_{ST} increased faster when the populations were increased in central Florida than in northern Florida. Genetic differentiation increased as the number of generations between each pair of populations increased (Figure 3-3).

The pairwise genetic distance (Nei, 1978) between G_0 and G_1C, G_2C or G_3C was 0.0080, 0.0141 and 0.0196, respectively (Table 3-3). The pairwise genetic distance between G_0 and G_1N, G_2N or G_3N was 0.0079, 0.0092 and 0.0142, respectively. These values again indicate a slight increase in population differentiation with each successive generation. The genetic distances between seed increase populations ranged from 0.0029 to 0.0133 with an average of 0.0082. The genetic distance between each population pair increased as the number of generations between each pair increased (Figure 3-4).

An UPGMA dendrogram was constructed from the matrix of genetic distances among these populations (Table 3-3). It clustered G_1C and G_1N with G_0 and the two G_2 populations with the two G_3 populations (Figure 3-5). This pattern of clustering suggests that a subtle but consistent genetic differentiation had occurred as seed increase progressed successively. The clustering of seed increase populations was mainly by generation rather than by seed increase site.

Principal Coordinate Analysis

Based on a total of 108 x 349 data points a matrix of Apostol (simple match) distances among 349 individuals from the seven populations were calculated in NTSYSpc and used to conduct a PCoA. Individuals of the G_0 and six increase populations were widely scattered, and no distinct grouping of any of the populations was observed in the PCoA plot (Figure 3-6). This distribution suggests that although it had occurred and was increasing over successive generations, the differentiation among the populations was still relatively weak.

Discussion

Genetic Differentiation between Seed Increase and Original Populations

In this study, the highest G_{ST} values (0.0513 and 0.0399) were observed between G_0 and the two advanced generations (G_3C and G_3N). These values were close to the G_{ST} value (0.046) detected among three other seed increase populations (SP1, SP2, and SP3) of *C. leavenworthii* using the AFLP marker system (Czarnecki et al., 2008). The three SP populations originated from a natural population in another county in central Florida (Orange County) and were increased under different climatic zones in Florida (Alachua, Gadsden or Pasco County) for one, two, or four generations. When the present and the previous studies were considered together, a total of nine seed increase populations of *C. leavenworthii* had been examined. The two studies suggest that there was only a very low level of genetic differentiation in *C. leavenworthii* seed production populations and that molecular markers were extremely powerful in revealing genetic differentiation before it became evident at the phenotypic level.

Among the major concerns in native forb seed increase are the potential of multiple seed increase locations, production practices and increase generations to cause significant differentiation in increase populations. In the current study, G_0 seeds were collected from central Florida, which was in USDA cold hardiness zone 9a and AHS heat zone 11; the northern Florida increase site was located in USDA cold hardiness zone 8b, AHS heat zone 9, while the central Florida increase site was in USDA cold hardiness zone 9b, AHS heat zone 10. Nevertheless, the G_{ST} values of northern Florida-increased populations were not greater than those of the central Florida-increased populations. These results suggest that *C. leavenworthii* seed may be increased either in central or northern Florida.

Slightly higher G_{ST} values were detected between the central Florida-increase populations (G_1C, G_2C, and G_3C). This was somewhat unexpected, because the central Florida seed increase site and the site where the G_0 seeds were collected shared a higher level of similarity in environmental conditions, especially in climatic conditions, including AHS heat zoning and USDA cold hardiness zoning. As described in the materials and methods, *C. leavenworthii* plants were grown in the ground beds at the northern Florida increase site while at the central Florida increase site the plants were grown in containers filled with artificial commercial potting substrate. This difference in production system between the two seed increase sites might have led to further differences in other aspects, such as fertility and soil moisture levels. Conceivably the container production system would be less similar than the ground bed production system to the soil conditions where the natural population was grown, and higher levels of growing condition dissimilarity might result in greater population differentiation or higher G_{ST} values. It remains to be determined if this was the actual cause of the higher G_{ST} values between central Florida-increased populations and the G_0 population.

The effect of increase generation on population differentiation was obvious (R^2 = 0.6299, P = <0.0001) in *C. leavenworthii*, as a linear relationship was detected between population-pairwise G_{ST} values and the generational differential between populations (Figures 3-3). The correlation between generational differential and genetic distances had the same effects (R^2 = 0.6428, P = <0.0001) (Figure 3-4). If the same rate of change is assumed for extended generations, the regression equation predicts that G_{ST} will remain below 0.10 even after eight consecutive generations of seed increase. It is

unknown whether or not this linear relationship and rate of G_{ST} change will continue for how many generations in *C. leavenworthii* seed increase. It seems plausible to assume that as seed increase approaches some unknown generations, the rate of G_{ST} change may slow down and the G_{ST} value will reach a plateau because there will be less genetic diversity in later generations causing less changes from generation to generation.

Possible Causes of Genetic Differentiation in *C. leavenworthii* Populations

Previously Czarnecki et al. (2008) reported a genetic shift in an introduced *C. leavenworthii* population (R1). The R1 population originated from central Florida (Orange County) and introduced to northern Florida using seeds increased for one generation in central Florida (SP2). When it was analyzed after growing for one year in northern Florida, R1 became more similar to northern Florida natural populations than to central Florida natural populations. It was suspected that gene flow from northern Florida local populations was the main cause of such genetic shift, because *C. leavenworthii* is an outcrossing, self-incompatible species and there were local natural populations in close proximity.

In the present study, there were four alleles that were not detected in G_0 but appeared in one or more of the increase populations, which suggests that gene flow from outside of the increase populations might have occurred. At both seed increase sites during the 3-year seed increase, no local natural populations were present close by, and residual *C. leavenworthii* and other *Coreopsis* plants from the soil seed bank were removed before they came into flowering. Thus the possibility of gene flow from outside sources could be ruled out. Two other possibilities seem to be more likely for the appearing of the four alleles. The G_0 individuals randomly selected for microsatellite

marker analysis were not carrying all the alleles present in the G_0 individuals used for seed increase. Evaluation of large populations is needed to accurately assess rare alleles. The second possibility is random genetic drift, which is known to occur frequently from generation to generation, especially when population sizes are small. Smaller populations have greater variation in gene frequency, where random changes in gene frequency can occur (Futuyma, 1979). It is likely that both factors might have contributed to the appearance of these alleles in seed increase populations because only a portion of the seed was used to produce each generation and was used for molecular analysis. Mutation is another possibility for the appearance of these different alleles.

Compared to the G_0 population, the increase populations had 6.7% to 16.3% fewer alleles present in the populations and more alleles in the populations with frequencies below 0.05. Further, there were two alleles amplified by the SSR marker COR7 that were present in the G_0 population but not in any of the increase populations. These results seem to suggest that allele loss and allele frequency change indeed occurred during seed increase. Several factors might have contributed to this observed allele loss and/or allele frequency change, including the founder effect (small population size typically due to a bottleneck), genetic drift, unintended selection, non-random mating, etc (Futumya, 1979). It is not known which factors and how much each factor affected this change in the alleles present in the seed increase populations. Because only a portion of the seed produced is used to produce the next generation, the founder effect and genetic drift is likely to occur. Unintended selection could have occurred

based on seed collection practices (early or late in the season) or seed germination practices that did not break dormancy of some seed.

Upholding the Genetic Integrity of *C. leavenworthii* through Seed Increase

In the PCoA plot the individuals from G_0 and the six increase populations were intermixed and did not form any definitive clustering by generation or location. This indicates that although some genetic changes (or shifts) had occurred, the increase populations remained very similar genetically to the original population. Overall the current molecular marker study confirms that the genetic integrity of *C. leavenworthii* was maintained during seed increase for up to three generations using the current production practices. It is likely that more generations could be produced from the original, natural population as indicated by the linear regression analysis. The linear regression equation also suggests that later generations should be assessed by common garden studies or molecular analysis to ensure that satisfactory level of genetic integrity is preserved.

Two similar studies assessed the genetic integrity of wheat and rye. Borner et al. (2000) determined that a high degree of genetic identity was upheld in up to 24 generations of multiplication in wheat using SSR markers. Chebotar et al. (2003) reported that the genetic integrity of two of the six rye accessions was upheld for 6-12 generations of multiplication using SSR markers. The other four accessions had significantly different allele frequencies after being multiplied for 7-13 times, which could be due to sample size or the lack of detection of rare alleles. These studies illustrate that the number of generations a species can uphold its genetic integrity depends on the species and population structure. It is important to use a large starting population

that represents a broad range of the alleles present in the population as well as large populations for producing each generation of seed increase.

Summary

SSR markers were highly polymorphic and detected an average of 10.4 alleles per primer pair in the G_0 population of *C. leavenworthii*. SSR markers also proved to be very powerful in revealing changes in alleles and allele frequencies. Total genetic diversity, G_{ST}, and genetic distance values changed slightly during seed increase at both locations when compared to the G_0 population. The largest differences were observed in the later generations (G_3) from both locations with the greatest G_{ST} value of 0.0513 and genetic distance of 0.0196. Genetic differentiation (G_{ST}) and genetic distance between populations increased linearly with the increase in generation. There did not appear to be a significant effect from producing seed in a different location from that of the natural population (G_0). The observed genetic changes did not seem to cause obvious genetic differentiation between the increase populations and the original population, which is congruent with the phenotypic data of the same *C. leavenworthii* populations. These results indicate that the overall genetic integrity of *C. leavenworthii* populations was upheld during three generations of seed increase. Current seed production practices can continue for *C. leavenworthii*, but later seed production populations should be evaluated to confirm the genetic integrity of *C. leavenworthii* seed increase populations.

Table 3-1. Primer sequences for 10 simple sequence repeat markers used to evaluate seven *C. leavenworthii* populations produced in northern and central Florida.

SSR markers	Primers	Primer sequences (5'→3')	Annealing temperature (°C)
Cor4	Forward	ACCCAATCCAATCCCTTCTC	50
	Reverse	TCATCGTTCGTGTGACACATT	
Cor7	Forward	GAGAGAACGGGGGAAAGAG	50
	Reverse	TTCCAATCCTAAATACCTAGAAACC	
Cor8	Forward	GTTCTTTGGGAGGGTGTTATCG	50
	Reverse	CATGGCATCACAAGCAGGTT	
Cor10	Forward	GAAGCCCAAAAGCCTAATTG	45
	Reverse	TTTCTCCTAGCTTTCCTGCTG	
Cor12	Forward	CTCACCCGTGATGTCGAGTT	45
	Reverse	ACATCTCACCCTCCCCTGAC	
Cor18	Forward	AAGCACACATAACCGCTCCT	50
	Reverse	TGCTCTCTGCCATGAATCAC	
Cor19	Forward	GGATCTCCTTCTTGCCTCCT	50
	Reverse	AGCCATAAACCCCAGATCCT	
Cor21	Forward	GAAAATGAGAAGACGAGGAA	45
	Reverse	AAGATGCTTTAACTTGACAGATT	
Cor23	Forward	CAGCTGGCCCATATCCTTCT	45
	Reverse	AGCTCGTCACAAAGGTTGAGG	
Cor65	Forward	GGCCACGTCTCCTTTTTACA	45
	Reverse	TTGAAATTGAAATGGGGTATGAG	

Table 3-2. The number of individuals, number of alleles and percentage of polymorphic loci used to evaluate the genetic differences in seven populations of *C. leavenworthii* produced in northern and central Florida.

Populations	G_0	G_1C	G_2C	G_3C	G_1N	G_2N	G_3N	Combined
Individuals surveyed (no.)	50	51	49	50	52	46	51	349
				Alleles detected				
COR4	11	10	7	7	11	9	11	12
COR7	14	11	11	11	9	11	10	14
COR8	8	8	7	6	6	8	9	9
COR10	6	6	5	4	6	4	5	6
COR12	17	16	14	11	18	16	13	18
COR18	4	5	4	4	4	4	4	5
COR19	13	11	13	13	13	13	13	13
COR21	13	13	12	13	13	11	12	13
COR23	14	13	14	14	13	14	14	14
COR65	4	4	4	4	4	4	4	4
Total	104	97	91	87	97	94	95	108
Pp (%)	96.1	88.2	83.3	78.4	88.6	88.7	88.2	

Table 3-3. Total genetic diversity (H_T), G_{ST}, and genetic distances for seven populations of *C. leavenworthii* produced in northern and central Florida.

	H_T	G_0	G_1C	G_2C	G_3C	G_1N	G_2N	G_3N
G_0	0.1736		0.0244[z]	0.0394	0.0513	0.0238	0.0282	0.0399
G_1C	0.1666	0.0080[z]		0.0382	0.0367	0.0127	0.0231	0.0336
G_2C	0.1558	0.0141	0.0133		0.0183	0.0354	0.0195	0.0130
G_3C	0.1593	0.0196	0.0128	0.0050		0.0342	0.0346	0.0143
G_1N	0.1706	0.0079	0.0030	0.0123	0.0119		0.0240	0.0292
G_2N	0.1590	0.0092	0.0069	0.0052	0.0114	0.0075		0.0244
G_3N	0.1541	0.0142	0.0113	0.0029	0.0034	0.0096	0.0072	

[z] G_{ST} values are above the diagonal and pairwise genetic distances (Nei, 1978) between populations are below the diagonal.

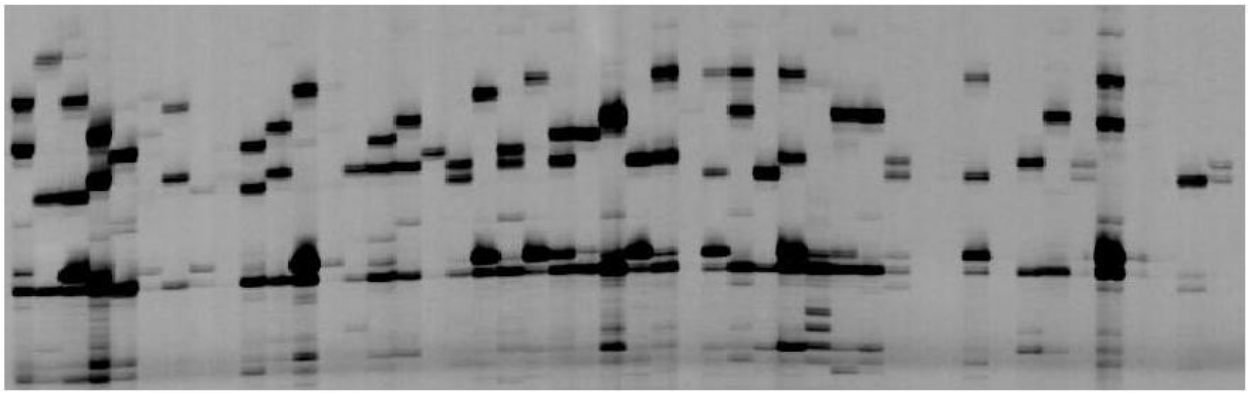

Figure 3-1. Gel image of simple sequence repeat marker bands (alleles) amplified from *C. leavenworthii* individuals by COR12 and detected by the LI-COR 4300 DNA Analyzer.

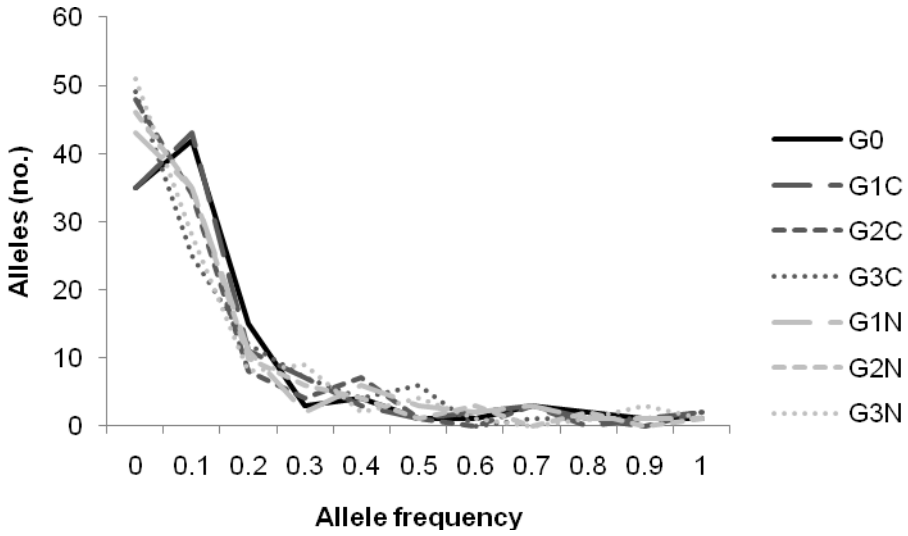

Figure 3-2. The number of SSR marker alleles with respect to their frequencies of occurrence in the *C. leavenworthii* source population (G_0), three populations increased in central Florida (G_1C, G_2C and G_3C) and three populations increased in northern Florida (G_1N, G_2N and G_3N).

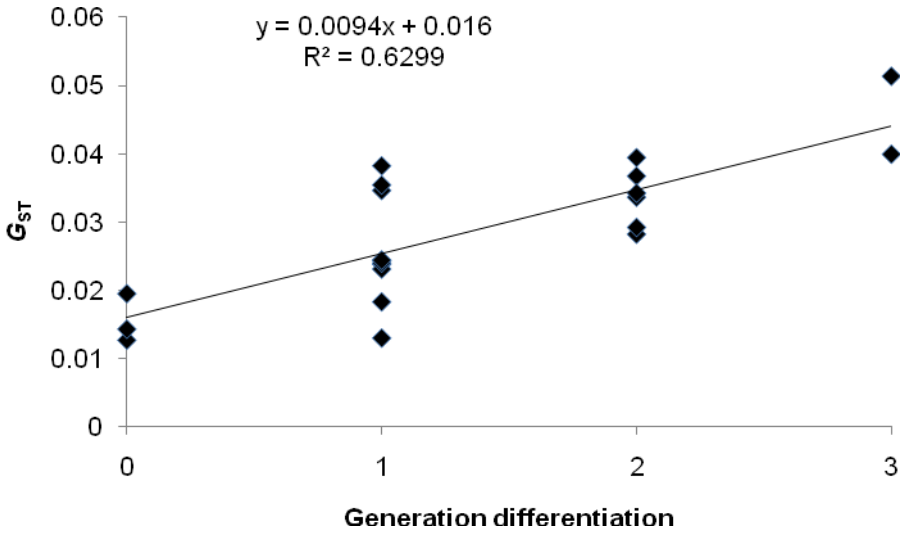

Figure 3-3. Regression of G_{ST} values when compared by the difference in the number of generations between each combination of seven *C. leavenworthii* populations.

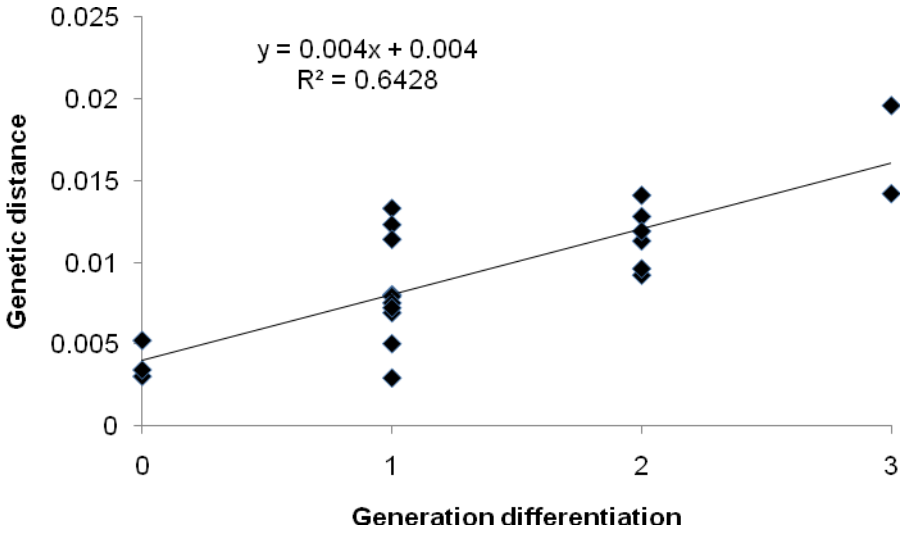

Figure 3-4. Regression of the genetic distances when compared by the difference in the number of generations between each combination of seven *C. leavenworthii* populations.

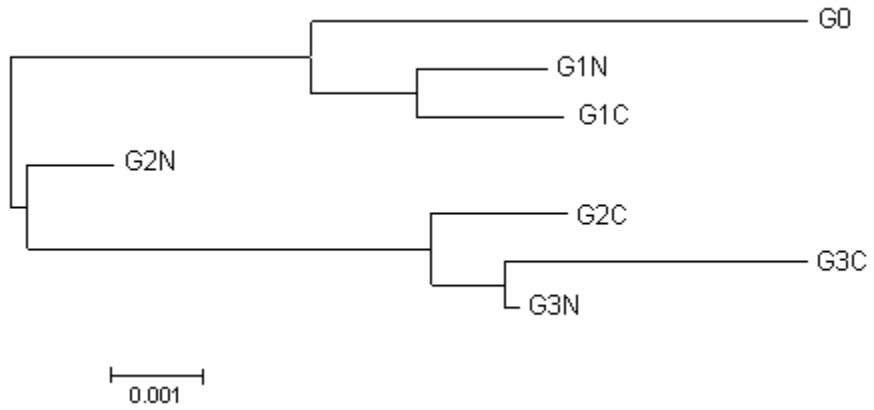

Figure 3-5. UPGMA dendrogram of the G_0 population and six seed increase populations (three from central Florida: G_1C, G_2C and G_3C; and three from northern Florida: G_1N, G_2N and G_3N) of *C. leavenworthii* populations based on their pairwise genetic distances.

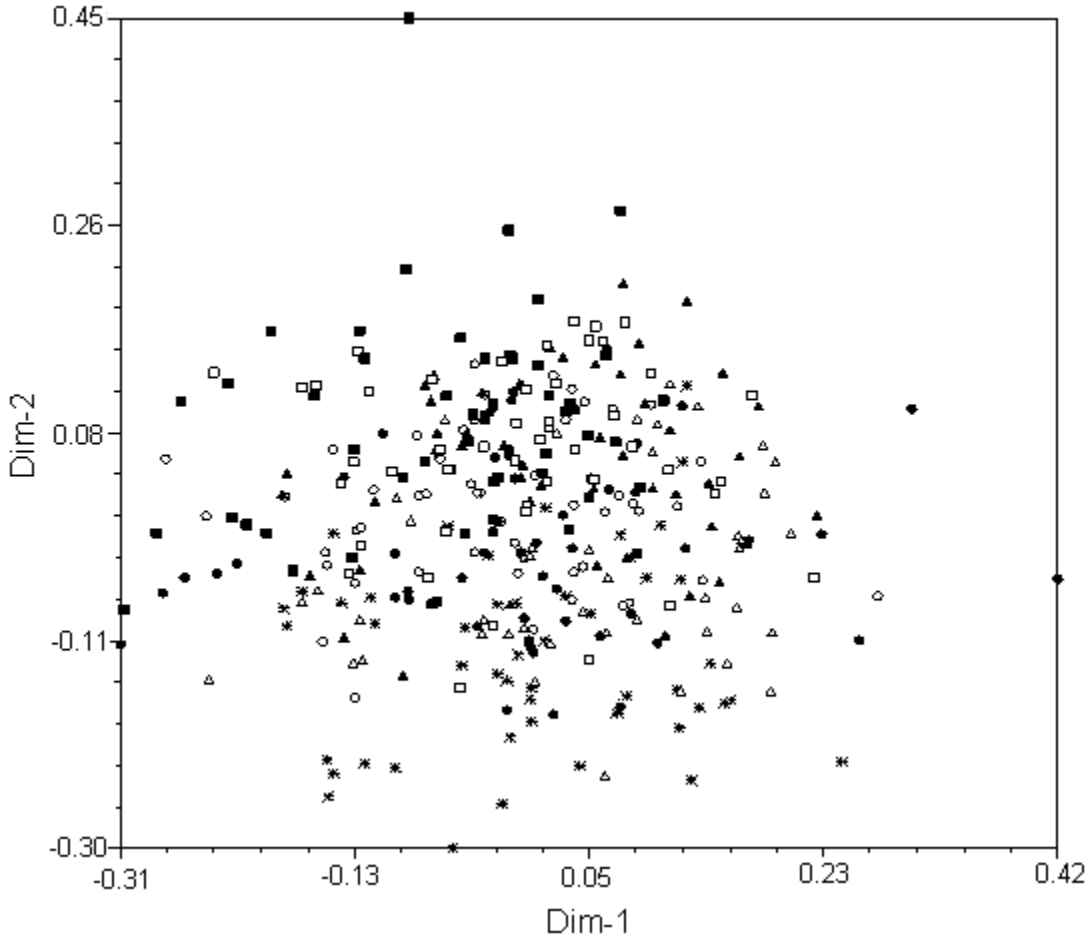

Figure 3-6. Plot of 50 individuals of the G_0 (ж) population and 299 individuals of six seed increase populations (● G_1C,▲ G_2C,■ G_3C,○ G_1N,Δ G_2N,□ G_3N) of *C. leavenworthii* based on the first two principal coordinates from 108 SSR alleles.

CHAPTER 4
INTERSPECIFIC HYBRIDIZATION BETWEEN *COREOPSIS LEAVENWORTHII* AND *COREOPSIS TINCTORIA* AND EFFECTS ON PROGENY GROWTH, DEVELOPMENT AND REPRODUCTION

Justification

Coreopsis is Florida's state wildflower and natural populations of *C. leavenworthii* grow throughout the state (USDA, 2011a) and along roadsides in highway beautification projects by the Florida Department of Transportation (FDOT). Because *C. leavenworthii* seed is solely produced in Florida and many other species of *Coreopsis* grow in Florida, it is important to prevent contamination from other *Coreopsis* species into natural and seed production *C. leavenworthii* populations. Most species are not compatible with *C. leavenworthii*, but *C. tinctoria* has been found to set interspecific hybrid seed with *C. leavenworthii* and the interspecific F_1 hybrids have 22% pollen stainability (Smith, 1976). *C. tinctoria* has been reported as growing in the northern part of Florida (Smith, 1983) and in some counties in Florida (USDA, 2011b). FDOT has also reported using *C. tinctoria* in highway beautification projects.

Because it is known that both species are growing in Florida (USDA, 2011a, 2011b) and they are cross compatible, information on the level of compatibility and the effects of interspecific hybridization is needed. This will help determine the fitness level of interspecific hybrids compared to each species and methods of prevention of gene flow and interspecific hybridization between these two species. Many studies have assessed the effects of interspecific hybridization by producing synthetic hybrids and evaluating the vegetative and reproductive characteristics of these interspecific hybrids. Frequently the F_1 generation does not exhibit all of the potential consequences of interspecific hybridization, so it is necessary to produce F_2 or later and backcross

generations if possible. The backcross population can be very useful to predict the effects of interspecific hybridization because in natural settings most of the next generation will result from backcross pollinations (Hufford and Mazer, 2003).

Arriola and Ellstrand (1997) found that synthetically produced interspecific F_1 hybrids between S*orghum halepense* and *S. bicolor* were not significantly different for the seven reproductive or vegetative characteristics evaluated compared to both parental species. Synthetic interspecific F_1 *Oryza rufipogon* and *O. sativa* hybrids were evaluated for eleven reproductive and vegetative characteristics during the entire life cycle of the crop and found that the hybrids had the same fitness level as the parental species (Song et al., 2004). The hybrids were slightly inferior in reproductive characteristics with lower seed set and pollen viability but showed greater fitness in vegetative characteristics, indicating that the interspecific hybrids were viable enough to persist in later generations allowing interspecific hybridization to continue. Several synthetic interspecific hybrid populations were produced using *Eucalyptus gunnii* as the maternal parent and *E. cordata*, *E. dalrympleana*, *E. viminalis*, *E. macarthurii*, *E. nitens*, *E. globulus* and *E. ovata* as the pollen sources (Potts et al., 1987). Although wide intra- and interspecific crosses were more likely to set seed in *Eucalyptus* rather than selfing and close intraspecific crosses, plants that were produced from selfing or close intraspecific crosses were more likely to survive than wide intra- and interspecific crosses. Plants that did survive from interspecific crosses were significantly taller and more vigorous than those from intraspecific crosses. These studies illustrate that interspecific crosses can produce vigorous and viable interspecific hybrids that can

persist in later generations, making the evaluation of *C. leavenworthii-C. tinctoria* hybrids important and critical.

The information known is limited, but interspecific hybridization could greatly affect natural and seed production *C. leavenworthii* populations by contamination for several generations. The objective of this study was to determine the fitness level of *C. leavenworthii-C. tinctoria* interspecific hybrids in the F_1 and F_2 generations by comparing them to intraspecific populations of *C. leavenworthii* and *C. tinctoria*. This will help determine if these interspecific hybrids are likely to persist in nature and affect pure populations of either species.

Materials and Methods

Seed Source

C. leavenworthii seed was collected by Nancy Bissett from a natural population at the Reedy Creek Mitigation Bank in Polk County, FL. *C. tinctoria* seed was purchased from Wildseed Farms in Fredericksburg, TX.

Interspecific Pollinations and Hybrid Population Development

Synthetic interspecific crosses were made in June, July and August 2007 to produce two F_1 hybrid populations, one (COLE F_1) using *C. leavenworthii* as the maternal parent and the other one (COTI F_1) using *C. tinctoria* as the maternal parent (Figure 4-1). Forty *C. leavenworthii* and thirty-five *C. tinctoria* plants were used to make each F_1 population, where flower buds on the seed parent plants, ten for COLE F_1 and five for COTI F_1, were bagged prior to pollination. Once the disk flowers of the maternal plants were mature on the flower head and anthesis was occurring, the pollen was removed from the flower head. The pollen source was collected by removing pollen from one flower head of each plant of the appropriate population into a plastic container.

The flower head of the maternal plant was dipped into the container with the appropriate pollen source. The flower head was rebagged and not removed until the seed head matured. After all of the seed for each seed parent was mature, it was collected and bulked per maternal parent and cross type, which will be referred to as a line from this point forward. The F_1 populations were self pollinated to produce two synthetic F_2 populations (COLE F_2 and COTI F_2) using the same pollination technique. Selfing was done between 3 and 25 November 2008. Parental plants used for both cross types were grown in 3.2-L containers filled with Vergrow verlite container mix A (Tampa, FL) mixed with 8.6 g of 15N-3.9P-10K (5-6 mo. Osmocote, The Scotts Co., LLC, Marysville, OH) per container in the greenhouse (29.4/23.9°C). Supplemental lighting was provided from 12:00 AM to 4:00 AM every morning until all seed was mature and collected from the plants.

Intraspecific Pollination and Reference Population Development

Synthetic *C. leavenworthii* intraspecific crosses (COLE REF) were made between 27 July and 5 August 2007 to produce the self population (Figure 4-1). *C. tinctoria* intraspecific crosses (COTI REF) were made between 7 and 10 August 2007 to produce the self population. The same *C. leavenworthii* and *C. tinctoria* plants were used to produce the intraspecific populations as the interspecific F_1 populations with five flower buds bagged for each cross type using the same pollination technique previously described.

Crossability

The number of seed produced per seed head was counted for three seed heads per plant per cross type from the hand pollinations completed in the greenhouse. Seed

germination tests were completed for both years by counting the number of seedlings that emerged for each population.

Assessing Progeny Growth, Development and Reproduction

Common garden studies were conducted in 2009 and 2010 to evaluate the effects of interspecific hybridization on the fitness of *C. leavenworthii*. In each year individuals from the various generations were grown under the same conditions in the greenhouse and subsequently in the field, and they were assessed on a number of vegetative and reproductive characteristics, including plant height, plant dry weight, days to flower, pollen stainability, seed production and seed germination.

Growing conditions

2009. Seed were sowed on 19 February 2009 into 148-mL cell Speedling flats (32-cell flats) (Sun City, FL) in Fafard 3B soil (Anderson, SC) media. The seed were placed on the surface of the soil and germinated under mist irrigation in the greenhouse (29.4/23.9°C). The seedlings were transplanted in 31-mL cell Speedling flats (128-cell flats) in Fafard 3B soil media on 14, 15 and 16 March. The plants were then transplanted in the field on 4 May. The field was a mix of Myakka, Haplaquents and St. Johns sandy soil. The beds were 25 cm high, 71 cm wide at the top and 81 cm wide at the base. Plants were spaced 55.9 cm apart in rows with two rows per bed. The rows in each bed were spaced 40 cm apart. The beds were fumigated with 67% methyl bromide: 33% chloropicrin at 197 kg ha^{-1}. The plants were fertilized with a total of 169 kg ha^{-1} of nitrogen, 37 kg ha^{-1} of phosphorus and 210 kg ha^{-1} of potassium throughout the year through drip irrigation.

2010. Seed were sowed on 20 January 2010 into 148-mL cell Speedling flats in Fafard 3B soil media. The seedlings were transplanted in 31-mL cell Speedling flats in

Fafard 3B soil media on 15 and 16 February. Plants were transplanted in the field in a single row 30.5 cm apart on 5 April. All field conditions were the same as the previous year except that no fertilization was applied and the plants were irrigated through seepage irrigation.

Data collection

Plant height and dry weight. Plant height was quantified in centimeters by measuring the tallest point for each plant growing in its respective year on 13 July 2009 and 2 June 2010. In 2009 plants were harvested from the field on 15-21 July, 72-78 days after field planting, and dried between 21.1-34.4°C. In 2010 plants were harvested from the field on 15-17 June, 71-73 days after field planting, and dried at 37.8°C and 20% relative humidity for 3-4 weeks before weight was recorded.

Days to flower. The number of days to flower was recorded from 23 April to 31 July 2009 and 28 April to 7 June 2010. The date was recorded for each plant in the field when the first flower opened and the number of days was counted from seed sowing.

Field seed production and seed germination. Five seed heads were collected from each plant in the field on 8 July 2009 and 2 and 3 June 2010 and the number of seed was counted. The seed collected in the field from 2009 were sowed for seed germination tests, where 100 seed collected from each plant in the field were sowed in 148-mL cell Speedling flats in Fafard 3B soil media for each block separately and the number of emerged seedlings was counted three weeks after sowing. All other seed germination conditions were the same as previously described.

Pollen stainability. For 2009 flower heads were collected in September and October. A total of ten flower heads were collected for each line (maternal parent and

cross type) prior to anthesis and bulked together for pollen staining. Flower heads were soaked in about 200-µL of lactophenol cotton blue stain (ENG Scientific Inc., Clifton, NJ) for about 3 h at room temperature. About 10-µL of stain was pipetted on the slide with a few ray flowers and a cover slip was placed on top of the stain. Three slides per line were prepared and pictures of four fields per slide were taken from the microscope. An average of 818 pollen grains per line was counted. Pollen grains were considered stainable if they were stained a deep, uniform blue and were very developed (large and plump).

Flowers heads were collected on 3 June 2010 and stained in lactophenol cotton blue stain (Fluka Analytical, Buchs, Switzerland) overnight at room temperature. One flower head per line (maternal parent and cross type) from each block was collected and bulked, resulting in eight flower heads per line. Three slides per line were prepared and pictures of three fields per slide were taken. An average of 258 pollen grains per line was examined. All other procedures for pollen staining were the same as the previous year.

Experimental design

Thirty seed per line were split into three replications of ten seeds per replication. Each replication was sowed into an individual cell of a 148-mL cell Speedling flat. There were 25 lines for each population in 2009 and 13 to 25 lines for each population in 2010. The plants were grown in a randomized complete block design in the field with 15 blocks in 2009 and 8 blocks in 2010 with 1 plant/line/block. A total of 375 plants in 2009 and 104-375 in 2010 were planted per cross type. All plants from all six cross types were randomized within each block.

Statistical analysis

The data points for each characteristic measured for each line were averaged by block. The average for each block was then used for statistical analysis. Significant differences were found for each data point evaluated by Analysis of Variance (ANOVA) using PROC GLM in SAS (SAS, 1997). Data were transformed using the Arcsine Square Root method for seed germination tests and pollen stainability. Based on the ANOVA test, further statistical analysis was performed using the Tukey W Procedure for mean separation analysis in SAS.

Results

Crossability between *C. leavenworthii* and *C. tinctoria*

On average the crosses among 40 *C. leavenworthii* individuals (COLE) produced 50.2 seed per seed head and the crosses among 35 *C. tinctoria* individuals (COTI) produced 14.9 seed per seed head (Figure 4-2). Thus, COLE produced 236.9% more seed per seed head than COTI, indicating a remarkable difference between the two species in seed production per seed head (Table 4-1). This difference seems to be largely due to the difference between the two species in the number of female florets that each flower head bears.

On average the interspecific crosses with *C. leavenworthii* being the seed parent (COLE x COTI) produced 61.9 seed per seed head, and the interspecific crosses with *C. tinctoria* being the seed parent (COTI x COLE) produced 25.0 seed per seed head (Figure 4-2). Thus, the COLE x COTI crosses produced significantly more seed (23.3%) than the COLE crosses, and the COTI x COLE crosses produced significantly more seed (67.8%) than the COTI crosses, indicating full crossability between the two species.

The seed germination rates for seed produced by hand pollinations were not significantly different between years (Table 4-1). The mean seed germination rates for COLE was 67.7% and for the COTI was 67.9%, which was not significantly different (Figure 4-3). The mean seed germination rates for the seed produced from crosses COLE x COTI was 69.7% and from crosses COTI x COLE was 71.7%, which were not significantly different from each other or the self pollinations. These results showed again that there were no biological factors preventing these interspecific seed from germination and that the two species were fully compatible.

Effects of Interspecific Hybridization on Plant Height

There were significant differences between years and among populations for plant height (Table 4-2). The mean plant height of the various populations in 2010 was significantly less than that in 2009: 10.4-14.2% reduction for the parental populations, 17.6-18.0% reduction for the F_1 populations and 9.7-11.1% reduction for the F_2 populations.

In 2009, the mean plant height of the COLE REF and COTI REF populations was 69.7 and 95.6 cm, respectively (Figure 4-4). In 2010, the mean plant height of the COLE REF and COTI REF populations were 59.8 and 85.6 cm, respectively. Thus, the plants of the COTI REF population were on average 25.9 cm (in 2009) or 25.8 cm (in 2010) taller than those of the COLE REF population.

In 2009, the mean plant height of the COLE F_1 population was 82.7 cm (Figure 4-4), similar to the mid-parent value (82.6 cm) (Table 4-3). The same trend was observed in 2010, where the mean plant height of COLE F_1 (67.8 cm) was similar to the mid-parent value (72.7 cm). The mean plant height for COLE F_1 was significantly greater than that of the COLE REF population and significantly less than that of the COTI REF

population in both years. The mean plant height for the COLE F_2 population was 77.6 cm in 2009 and 70.1 cm in 2010, similar to the mid-parent values and the values of the COLE F_1 population in both years.

Overall, similar results were observed for the COTI F_1 and COTI F_2 populations as compared to the mid-parent values, except for that in 2009 the COTI F_1's value seemed to be greater than the mid-parent value (Table 4-3), indicating no effect due to interspecific hybridization (Table 4-4). In 2009, COTI F_1 and COTI F_2 populations had a mean plant height of 91.8 and 89.4 cm, respectively, and in 2010, they had a mean plant height of 75.6 and 79.5 cm, respectively (Figure 4-4).

When the two F_1 and F_2 populations were compared, the mean plant heights of the COTI F_1's were 11.0% (in 2009) to 11.5% (in 2010) taller than the COLE F_1's; the COTI F_2's were 15.2% (in 2009) to 13.4% (in 2010) taller than the COLE F_2's. These differences were consistent for the F_1 to F_2 populations and indicate a maternal effect from *C. tinctoria* on the plant height of its hybrids with *C. leavenworthii*.

Effects of Interspecific Hybridization on Plant Dry Weight

There were no significant differences between years for plant dry weight, so the data from the two years were combined for determining the effects of interspecific hybridization on plant dry weight (Table 4-2). The mean plant dry weight for COLE REF (0.083 kg) was significantly less than COTI REF (0.156 kg) (Figure 4-5). Thus on average the plant dry weight of the COTI plants were 88.0% greater than that of the COLE plants, imposing a maternal effect on the F_1 and even F_2 generations. The plant weight of COTI F_1 was 21.3% greater than COLE F_1, and the plant weight of COTI F_2 was 21.7% greater than COLE F_2's value. The maternal effect seemed to be consistent from F_1 to F_2.

A heterotic effect (Table 4-4) was present in F_1 populations, particularly in the COTI F_1 population. The plant dry weight of COLE F_1 was 0.136 kg (Figure 4-5), 4.6% greater than the mid-parent value (0.130 kg per plant) (Table 4-3). Whereas the plant weight of COTI F_1 was 0.165 kg, which is 26.9% greater than the mid-parent value, even 5.8% greater than the high parent value, which was COTI REF (0.156 kg).

The mean plant dry weight for COLE F_2 was 0.115 kg (Figure 4-5). This is only 84.5% of the mid-parent value (Table 4-3) and 84.6% of the COLE F_1's value, indicating a breakdown in plant dry weight in the F_2 generation. A similar breakdown seemed to be present in the COTI F_2 population (Table 4-4). The COTI F_2's plant dry weight value (0.140 kg) was only 84.8% of its F_1's value although the F_2's value was still 7.7% greater than the mid-parent value.

Effects of Interspecific Hybridization on Days to Flower

There were significant differences among populations and between years for the number of days to flower (Table 4-2). The mean number of days to flower of the various populations in 2010 was significantly greater than that in 2009: 6.0-8.3% increase for the parental populations, 0.3-4.7% increase for the F_1 populations and 6.3-7.8% increase for the F_2 populations.

In 2009 and 2010 COLE REF (107.3 and 113.7 days, respectively) took significantly fewer days to flower than COTI REF (114.2 and 123.7 days, respectively) (Figure 4-6). The plants of the COTI REF population took on average 7.0 (in 2009) and 10.0 (in 2010) days longer to flower than those of the COLE REF population.

There seemed to be an interaction between year and the effect of interspecific hybridization. In 2010 when the plants were grown in spring to summer, the mean number of days to flower for the COLE F_1, COLE F_2, COTI F_1 and COTI F_2 populations

were 117.4, 119.9, 120.5, 121.9 days, respectively (Figure 4-6), all similar to the mid-parent value (117.6 days) (Table 4-3) and not showing any heterotic, hybrid breakdown or maternal effects. However, when the plants were grown in 2009, the two F_1 populations (COLE F_1 and COTI F_1) and one F_2 population (COTI F_2) had their mean number of days to flower between 117.1 and 114.2 days, greater than their mid-parent value (110.8 days). The only exception was the COLE F_2 population, whose number of days to flower was 111.2 days. Thus in 2009, the F_1 and F_2 populations showed a heterotic effect (Table 4-4).

Effects of Interspecific Hybridization on Pollen Stainability

There were significant differences found between years and among populations in 2009 for pollen stainability (Table 4-2). In 2009 the mean pollen stainability for COLE REF (20.1%) and COTI REF (16.7%) were not significantly different (Figure 4-7). The mean pollen stainability for COLE F_1 was 3.8% and for COTI F_1 was 6.9%. Thus, the interspecific hybrids had pollen stainabilities significantly less than COLE REF's and COTI REF's values and the mid-parent value (18.4%) (Table 4-3). The mean pollen stainability for COLE F_2 was 6.1% and for COTI F_2 was 7.8%, which was less than the mid-parent value, significantly lower than COLE REF's and COTI REF's values but similar to COLE F_1's and COTI F_1's values. The significant decrease for the mean pollen stainability for the COLE F_1 and F_2 and COTI F_1 and F_2 populations from the COLE REF and COTI REF populations indicated a reduction possibly due to chromosome mispairing in the interspecific progeny of *C. leavenworthii* and *C. tinctoria* (Table 4-4).

The mean pollen stainability of the parental populations in 2010 was significantly less than that in 2009 (17.4-32.3%). In 2010 none of the populations were significantly

different for pollen stainability (Table 4-2). The mean pollen stainability for COLE REF and COTI REF was 3.5% and 5.4%, respectively (Figure 4-7). The mean pollen stainability for COLE F_1 and COTI F_1 was 5.5% and 4.0%, respectively. The mean pollen stainability for COLE F_2 and COTI F_2 was 4.5% and 2.7%, respectively. The F_1 and F_2 populations from both species were similar to the mid-parent value (4.5%) (Table 4-3).

Effects of Interspecific Hybridization on Seed Production and Seed Germination

Seed production of hand-pollinated F_1 populations

The mean number of seed produced per seed head for the COLE x COTI F_1 pollinations was 35.3 seed (Figure 4-2), which was 70.3% of that of the COLE pollinations and 57.0% of that of the COLE x COTI pollinations. The COLE x COTI F_1 pollinations produced significantly less seed per seed head than the COLE and COLE x COTI pollinations. The mean number of seed produced by the COTI x COLE pollinations was 31.9 seed, which was 214.1% of that of the COTI pollinations and 127.6% of that of the COTI x COLE pollinations. The COTI x COLE F_1 pollinations produced significantly more seed than the COTI pollinations and not significantly different amounts of seed than the COTI x COLE pollinations.

Seed production of open-pollinated F_1 and F_2 populations

There were significant differences for seed production from open pollinations in the field between years and among the populations (Table 4-2). The mean seed production from open pollinations in the field of the various populations in 2010 was significantly greater than that in 2009: 31.9-44.3% increase for the parental populations, 51.0-78.8% increase for the F_1 populations and 41.8-77.1% increase for the F_2 populations.

In 2009 COLE REF produced significantly more seed (476.0 seed) than COTI REF (416.2 seed) (Figure 4-8). In 2010 COLE REF again produced more seed (627.9 seed) than COTI REF (600.4 seed), but the difference (27.5 seed) was not significant.

The mean seed production for COLE F_1 was 294.0 seed in 2009 and 525.8 seed in 2010 (Figure 4-8), which was less than the mid-parent value (446.1 and 614.1 seed, respectively) (Table 4-3). The mean seed production for COLE F_1 was significantly less than COLE REF and COTI REF in 2009 and was significantly lower than COLE REF and not significantly different than COTI REF in 2010. The mean seed production for COLE F_2 was 309.7 seed in 2009 and 548.6 seed in 2010, which was less than the mid-parent value for both years. The mean seed production for COLE F_2 was not significantly different from COLE F_1 but was significantly less than COTI F_1 in 2009 and was not significantly different than COLE F_1 and COTI F_1 in 2010.

The mean seed production for COTI F_1 was 336.9 seed in 2009 and 508.7 seed in 2010 (Figure 4-8), which was significantly less than COLE REF and COTI REF for both years and less than the mid-parent value (Table 4-3) for both years. The mean seed production for COTI F_2 was 378.8 seed in 2009 and 537.2 seed in 2010, which was less than the mid-parent value for both years. The mean seed production for COTI F_2 was significantly greater than COLE F_1 and not significantly different from COTI F_1 in 2009 and not significantly different than COLE F_1 and COTI F_1 in 2010.

Although seed production was greater in 2010, both years were similar, where the parental populations were greater than the F_1 and F_2 populations and the F_2 populations increased slightly from the F_1 populations, suggesting chromosome mispairing and

dilution (Table 4-4) in the F_1 and F_2 populations with slight recovery in the F_2 populations.

Seed germination of the hand-pollinated F_1 population

The mean seed germination rate for the seed produced by COLE x COTI pollinations was 79.1%, which was significantly greater than the COLE and COLE x COTI hand pollinations (Figure 4-3). The mean seed germination rate for the seed produced by hand in the COTI x COLE F_1 pollinations was 83.0%, which was significantly greater than the COTI and COTI x COLE hand pollinations.

Seed germination of open-pollinated F_1 and F_2 populations

There were significant differences among populations for seed germination rates from the seed collected from open pollinations in the field in 2009 (Table 4-2). The mean seed germination rate for COLE REF (42.9%) was significantly lower than COTI REF's value (55.7%) (Figure 4-9). On average the seed germination rate for COLE F_1 was 49.7% and COTI F_1 was 50.9%, not significantly different than COLE REF's or COTI REF's value and similar to the mid-parent value (49.3%) (Table 4-3). The mean seed germination rate for COLE F_2 was 46.4% and for COTI F_2 was 49.3%, both similar to the mid-parent value and COLE F_1's and COTI F_1's values. The F_1 and F_2 populations were not significantly different from either parental population, indicating that interspecific hybridization had no obvious effect on seed germination (Table 4-4).

Discussion

Full Compatibility between *C. leavenworthii* and *C. tinctoria*

Both interspecific hand pollination crosses (COLE x COTI and COTI x COLE) produced more seed per seed head than the COLE and COTI hand pollinations when comparing the maternal parents. *C. leavenworthii* plants produced more seed per seed

head from the hand pollinations compared to *C. tinctoria* possibly because there were more disk flowers per flower head in *C. leavenworthii* than *C. tinctoria*. Because of this, there appeared to be a maternal effect for seed production for the synthetic hand pollinations. As previous work by Smith (1976) has shown, *C. leavenworthii* and *C. tinctoria* are highly compatible. When Parker (1973) made interspecific hybrid crosses between *C. leavenworthii* and *C. tinctoria*, 29 F_1 plants were recovered from one seed head and in the current study about 30.5 F_1 plants were recovered per seed head.

Effects of Interspecific Hybridization

In general there are two effects of interspecific hybridization, heterosis and outbreeding depression (Hufford and Mazer, 2003). Heterosis occurs when one generation of hybridization results in increased fitness in the F_1 generation due to increased heterozygosity but will slightly decline in later generations due to increased homozygosity. This was detected for number of days to flower (2009) in the current study (Table 4-4). Outbreeding depression is the reduction in mean population fitness between genetically distinct populations relative to parental populations. There are two types of outbreeding depression, hybrid breakdown and dilution. When hybrid breakdown occurs, there is a reduction in fitness due to the disruption of co-adapted gene complexes. The F_1 generations appeared to be exhibiting heterosis in the F_1 generation and hybrid breakdown in the F_2 generations for plant dry weight. Dilution is the reduction in fitness caused by the expression of only one half of the alleles of each parent, which appeared to affect seed production.

Heterosis

Days to flower (in 2009) appeared to be undergoing heterosis because the values of both characteristics were greater than the mid-parent value. Arriola and Ellstrand

(1997) found no difference in the mean number of days to flower between interspecific hybrid and parental plants when *S. bicolor* and *S. halepense* were crossed, where there were not significant differences for either parental species or the interspecific hybrids. These studies indicate that the effects on plant development of interspecific hybrids can change depending on the species studied.

Heterosis followed by hybrid breakdown

Plant dry weight appeared to be undergoing heterosis followed by hybrid breakdown because there was an increase in the F_1 generation and then a decrease in the F_2 generation. Similarly, Arriola and Ellstrand (1997) found no difference in aboveground biomass between the interspecific hybrids and parental populations for crosses between *S. bicolor* and *S. halepense*.

Chromosome mispairing and outbreeding depression

Pollen stainability reduced drastically in the F_1 and F_2 generations in 2009. In 2010 the percentage of stainable pollen was very similar among the populations. Seed production in the field was undergoing outbreeding depression because of reductions in both years. Both of these reductions were due at least partially to chromosome mispairing producing nonviable pollen and a reduced number of seed (Parker, 1973; Smith, 1976). The reduction in seed production was partially due to dilution because of the difference in number of disk flowers between *C. leavenworthii* and *C. tinctoria* and their interspecific hybrids.

Arriola and Ellstrand (1997) found much variation in pollen stainability in interspecific hybrid (0-98%) and parental populations (0-92%), indicating no effect on interspecific hybridization on pollen stainability. The same wide range was found for seed production per panicle between the interspecific hybrid and parental populations,

again indicating no effect from interspecific hybridization. Pollen viability, determined by pollen germination tests, and seed set was significantly lower for the interspecific hybrids than either parental species, where *O. sativa* had significantly higher pollen viability and seed set than *O. rufipogon* when these two species were crossed (Song et al., 2004). These studies indicate that the effects on pollen viability and seed production of interspecific hybrids can change depending on the species studied.

No effects detected on plant height and seed germination

Plant height was not affected by interspecific hybridization because the averages for the F_1 and F_2 populations were similar to the mid-parent values for both years. Seed germination did not appear to be affected by interspecific hybridization compared to the parental populations. All of the F_1 and F_2 populations had seed germination rates between those of the parental populations.

Maternal Effects on Interspecific Hybridization

The vegetative characteristics, plant height and dry weight, and days to flower were affected by the maternal species. When *C. leavenworthii* was used as the maternal parent, plant height was consistently lower compared to the crosses where *C. tinctoria* was the maternal parent. This occurred for all three cross types (intraspecific, F_1 and F_2) over both years. The *C. leavenworthii* populations were consistently lower than the *C. tinctoria* populations for plant dry weight, indicating maternal effects. When *C. leavenworthii* was used as the maternal parent, the individuals of these populations on average took fewer days to flower compared to *C. tinctoria* as the maternal parent in 2009, indicating maternal effects. In 2010 both the F_1 and F_2 populations had similar values to the mid-parent value, not showing any maternal effects. The only exception to

this was for COLE F_1 and COTI F_1. The other characteristics evaluated did not indicate maternal effects.

Effects of Differences in Environmental Conditions

The characteristics showed similar trends over both years but the values were quite different for each characteristic, most likely due to differences in the environmental conditions over both years. The fertilization rate and irrigation methods used between the two years were very different. In 2009 drip irrigation with fertilizer was applied to the plants. In 2010 supplemental fertilization was not used and the plants were irrigated by seepage irrigation. The growth and development of the plants in 2009 could have resulted from competition for light, nutrients and water because the plants were grown in two rows per bed instead of a single row as in 2010. There was not much difference in temperature or humidity over both years although both conditions were slightly higher in 2009 (Table 4-5). This most likely did not affect the growth and development of the plants.

The other major effect on the growth and development of the plants is the differences in day length between the two years. It took the plants a fewer number of days to flower in 2009 compared to 2010 most likely due to the fact that on average the plants were growing under longer day length conditions in 2009 compared to 2010 (Table 4-6). Because *Coreopsis* is a photoperiod sensitive crop, the number of hours of darkness affects the number of days to flower. *Coreopsis* plants flower under long day (short night) conditions, so the plants flower when night length drops below a certain amount of time.

When the population by year (genotype by environment) interactions were tested, the interactions for all of the traits evaluated were significant (Table 4-2). This indicated

that the environment affected the phenotype of the individuals and this was different for each year, indicating that environment had a strong affect on the traits evaluated.

Differences in Pollen Stainability and Seed Production over Two Years

High temperatures and relative humidities at anthesis can have a detrimental effect on Asteraceae pollen vitality (Hoekstra and Bruinsma, 1975). Smith (1976) reported *C. leavenworthii* and *C. tinctoria* interspecific hybrids had an average pollen stainability of 22%. The hybrids were grown in the greenhouse when pollen was collected at anthesis, whereas the plants in the current study were grown in the field where many environmental factors in the field are different and can have a detrimental effect on pollen viability and can change from season to season. Pollen viability appears to be higher in the greenhouse (personal observation) because the environmental factors, such as temperature and humidity, are easier to control and stabilize. The differences in the environmental conditions between the two years in the current study caused the differences in pollen viability between the two years including the time of year that the pollen was collected. Pollen was collected in late September and early October in 2009 and early June in 2010. Day length, temperature and humidity can be very different in these months. Although on average the environmental conditions were similar, it is difficult to determine the time frame of changes in the environmental conditions that could have affected pollen viability. The individuals in 2010 had dehiscent pollen and many bees visiting the flowers. In 2009 the pollen on the flower heads of the individuals did not noticeably dehisce. Because of this difference, it is possible that the insects “robbed” the pollen from the flower heads in 2010 before flower head collection for the pollen stainability tests. It has been reported that cotton blue can overestimate pollen stainability (Archibald et al., 2005). However, it

seems to be an accurate stain for *Coreopsis* because stained pollen grains normally appear dark, plump and much larger in size than unstained pollen grains that are small and shrunken. Although there was a lower frequency of stainable pollen in 2010, more seed per seed head was produced in the field in 2010 compared to 2009. The plants had more flowers and a longer flowering period in 2010 compared to 2009 (personal observation). There appeared to be more insects, especially Hymenoptera, in the field during 2010 compared to 2009. This could be due to the differences in crops planted around the *Coreopsis* plants. The main difference between the two years is in 2009 there were many crops for insects to extract nectar and transfer pollen. In 2010 there were not many crops around the *Coreopsis* plants, so the insects most likely fed off the *C. leavenworthii* plants.

Summary

Because COLE x COTI and COTI x COLE produced more seed than COLE and COTI, respectively, these two species are fully compatible. This indicates that interspecific hybridization could occur under natural conditions and persist over multiple generations. Number of days to flower had heterotic effects in the *C. leavenworthii-C. tinctoria* interspecific hybrids. Plant dry weight of the *C. leavenworthii-C. tinctoria* interspecific hybrids appeared to be affected by heterosis in the F_1 generation followed by hybrid breakdown in the F_2 generation. Pollen stainability and seed production were reduced in the F_1 and F_2 generations compared to the parental populations possibly due to chromosome mispairing and dilution for seed production. This also indicates that the *C. leavenworthii-C. tinctoria* interspecific hybrids are vigorous and may increase in viability over several generations, which would allow them to persist in later generations in a natural setting. Finally, the environmental conditions under which the plants are

grown affected the growth and development of the plants, indicating that growth and development differences can occur between seasons and years resulting in different effects from interspecific hybridization. This helped determine the fitness level of interspecific hybrids compared to intraspecific populations of both parental species. Because there was some outbreeding depression found, gene flow between these two species should be prevented. More generations could be created to assess the growth and development, as well as backcross generations. *C. leavenworthii-C. tinctoria* interspecific hybrids made in a natural setting should be assessed for growth and development and persistence with natural, pure populations of both species.

Table 4-1. Analysis of variance for the number of seed produced per seed head and seed germination from *C. leavenworthii* and *C. tinctoria* hand pollinations to produce six populations at the Gulf Coast Research and Education Center, Wimauma, FL in 2007 and 2008.

	Among populations			Between years	
Plant characteristic	Sample size	F value	Probability	F value	Probability
Seed production	75	46.36	0.0001	NA	NA
Seed germination	375	9.78	0.0001	0	0.9732

Table 4-2. Analysis of variance for several morphological and reproductive characteristics of six populations of *C. leavenworthii* and *C. tinctoria* grown at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010.

	Among populations			Between years		Population*year	
Plant characteristic	Sample size	F value	Probability	F value	Probability	F value	Probability
Plant height	375/200[z]	50.83/28.08	0.0001/0.0001	165.54	0.0001	2.78	0.0184
Plant dry weight		35.78[y]	0.0001	0.05	0.8220	3.99	0.0017
Days to flower		23.93/18.18	0.0001/0.0001	153.68	0.0001	8.73	0.0001
Pollen stainability		9.26/1.11	0.0001/0.3558	37.10	0.0001	5.88	0.0001
Seed production		32.64/6.63	0.0001/0.0001	453.73	0.0001	3.28	0.0069
Seed germination		3.08[x]	0.0119	NA	NA	NA	NA

[z]Value for 2009 year listed before the slash and 2010 after (2009/2010).
[y]2009 and 2010 years were not significantly different, so values for both years were combined.
[x]Data taken for only 2009 year.

Table 4-3. Comparisons of F_1 and F_2 average values to mid-parent values for each characteristic evaluated for *C. leavenworthii* and *C. tinctoria* interspecific hybrids.

	2009			2010		
Plant characteristic	Mid-parent	F_1	F_2	Mid-parent	F_1	F_2
Plant height (cm)	82.6	87.3	83.5	72.7	71.7	75.1
Plant dry weight (kg)	0.14	0.16	0.12	0.12	0.14	0.14
Days to flower	110.8	116.1	112.9	117.6	118.9	120.9
Pollen stainability (%)	18.4	5.4	7.0	4.5	4.8	3.6
Seed production (field) (No.)	446.1	315.5	344.3	614.1	517.2	542.9
Seed germination (field) (%)	49.3	50.3	47.8	NA	NA	NA

Table 4-4. Comparison of *C. leavenworthii* and *C. tinctoria* intraspecific and interspecific F_1 and F_2 populations.

Plant characteristic	COLE vs. COTI	F_1	F_2	Maternal effect
Plant height	COTI 25.8-25.9 cm taller	≈Mid-parent value	≈Mid-parent value	Yes
Plant dry weight	COTI 88.0% heavier	Heterosis	≈Mid-parent value	Yes
Days to flower	COTI took 7-10 days longer to flower	Heterosis in 2009	Heterosis in 2009	Yes 2009/No 2010
Pollen stainability	Similar	Chromosome mispairing	Chromosome mispairing	No
Seed production	COLE produced 27-60 more seed per 5 seed heads	Dilution	Dilution	No
Seed germination	COLE ~13% reduced seed germination	≈Mid-parent value	≈Mid-parent value	No

Table 4-5. Temperature and relative humidity averages throughout the growing year at the Gulf Coast and Education Center, Wimauma, FL in 2009 and 2010.

Year	AVG Temp (°C)	AVG min (°C)	AVG max (°C)	Relative Humidity (%)
2009	25.9	20.8	32.4	80.29
2010	24.4	18.2	31.3	76.51

Table 4-6. Day length averages throughout the growing year at the Gulf Coast and Education Center, Wimauma, FL in 2009 and 2010.

Year	Overall (h)	Prior to 1st flower (h)	Flowering period (h)	1 mo. Prior to and including flowering period (h)
2009	13.11	12.18	13.69	13.44
2010	12.35	11.84	13.59	13.23

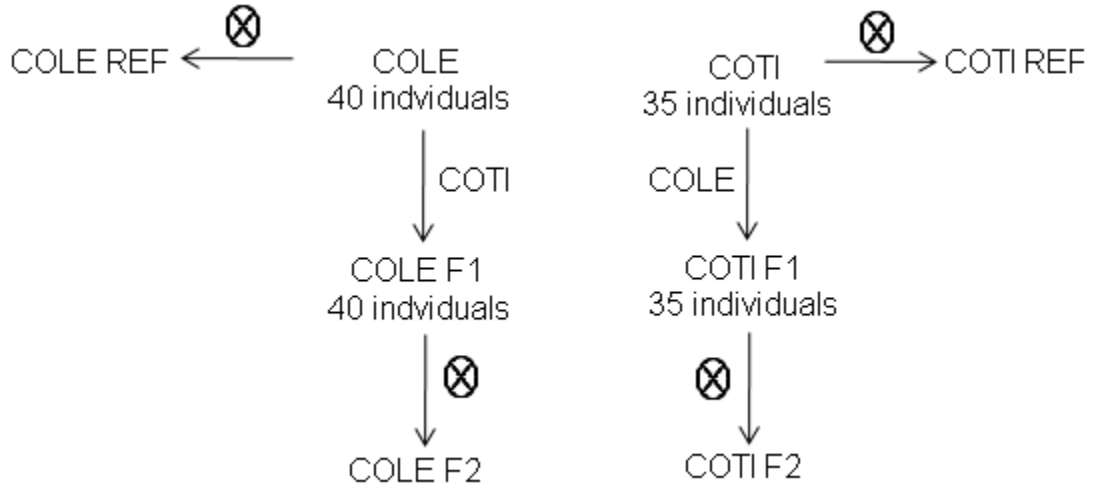

Figure 4-1. Crosses to produce populations to compare the interspecific F_1 and F_2 populations to intraspecific populations of *C. leavenworthii* and *C. tinctoria*.

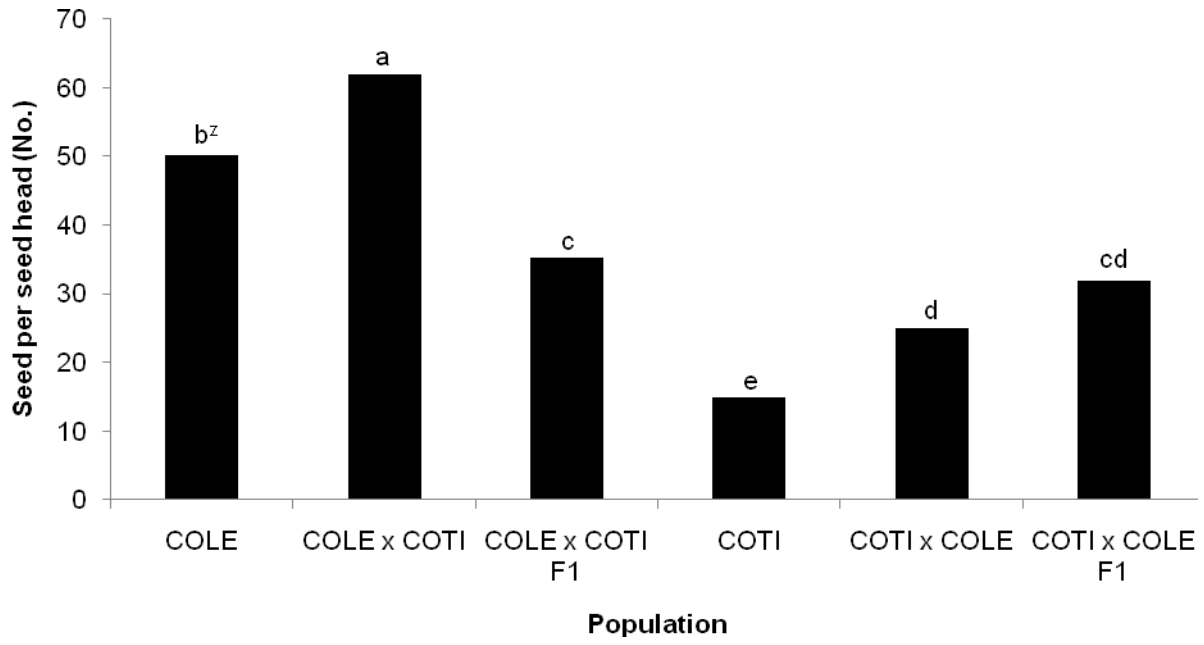

Figure 4-2. Differences in the amount of seed produced per seed head by hand pollinations of six populations of *C. leavenworthii* and *C. tinctoria* produced at the Gulf Coast Research and Education Center, Wimauma, FL in 2007 and 2008. [z]Means not followed by same letter are significantly different by Tukey's W Procedure at p≤0.05.

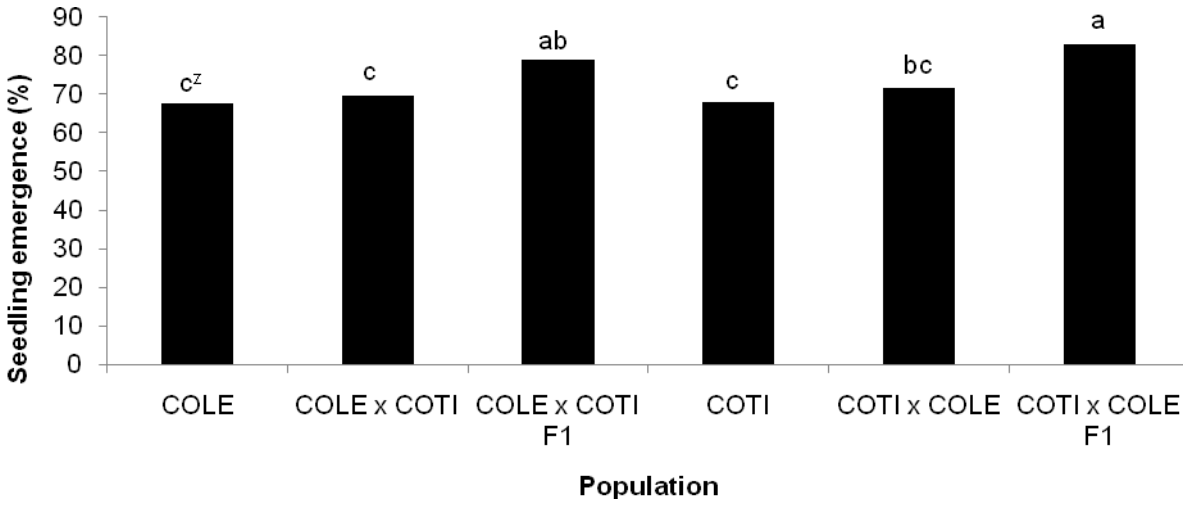

Figure 4-3. Differences in the seed germination rate of seed produced from hand pollinations of six populations of *C. leavenworthii* and *C. tinctoria* produced at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010. [z]Means not followed by same letter are significantly different by Tukey's W Procedure at p≤0.05.

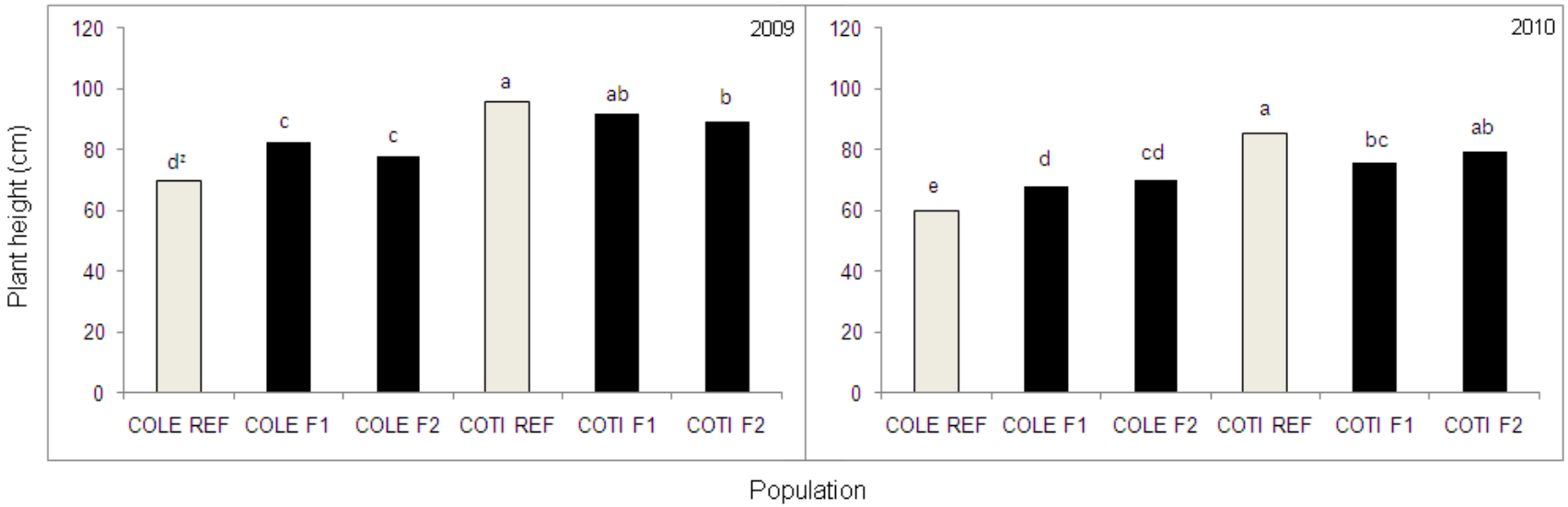

Figure 4-4. Differences in plant height (cm) of six populations of *C. leavenworthii* and *C. tinctoria* produced at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010. [z]Means within year not followed by same letter are significantly different by Tukey's W Procedure at p≤0.05.

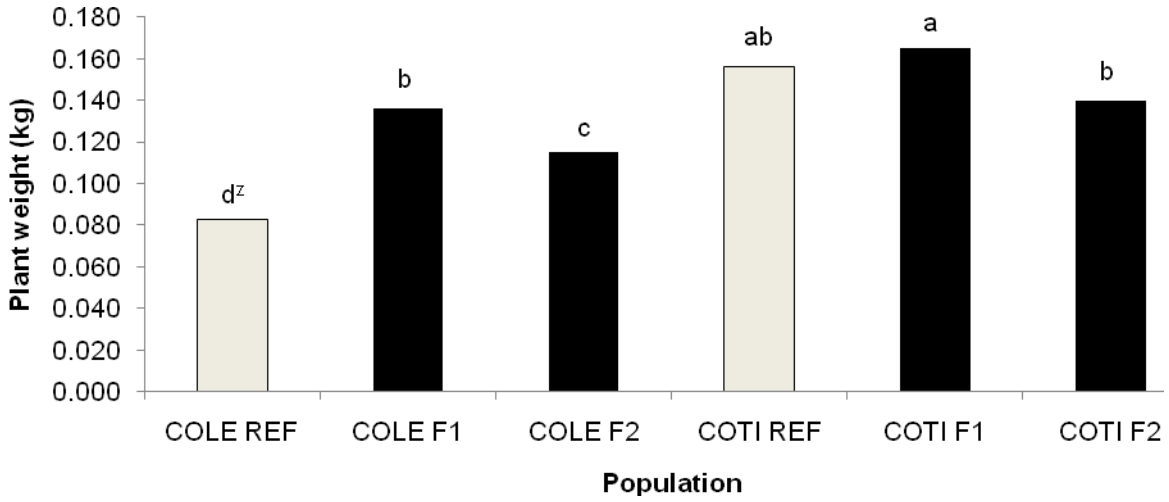

Figure 4-5. Differences in plant dry weight (kg) of six populations of *C. leavenworthii* and *C. tinctoria* produced at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010. [z]Means not followed by same letter are significantly different by Tukey's W Procedure at p≤0.05.

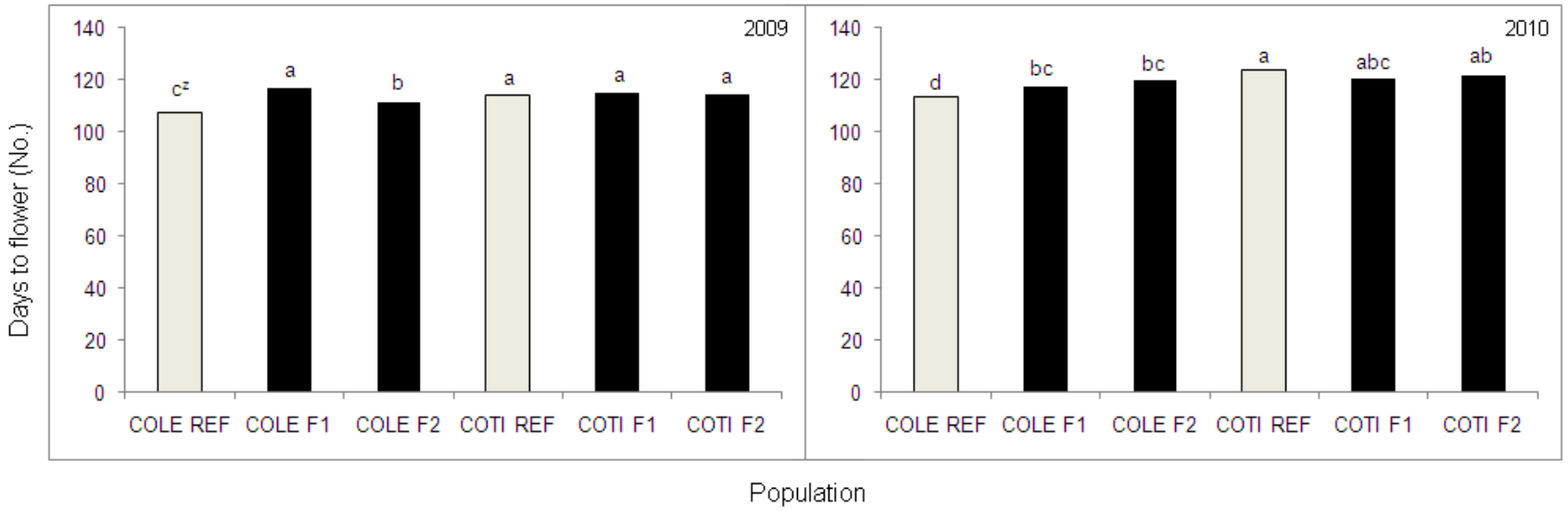

Figure 4-6. Differences in the number of days to flower of six populations of *C. leavenworthii* and *C. tinctoria* produced at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010. [z]Means within year not followed by same letter are significantly different by Tukey's W Procedure at p≤0.05.

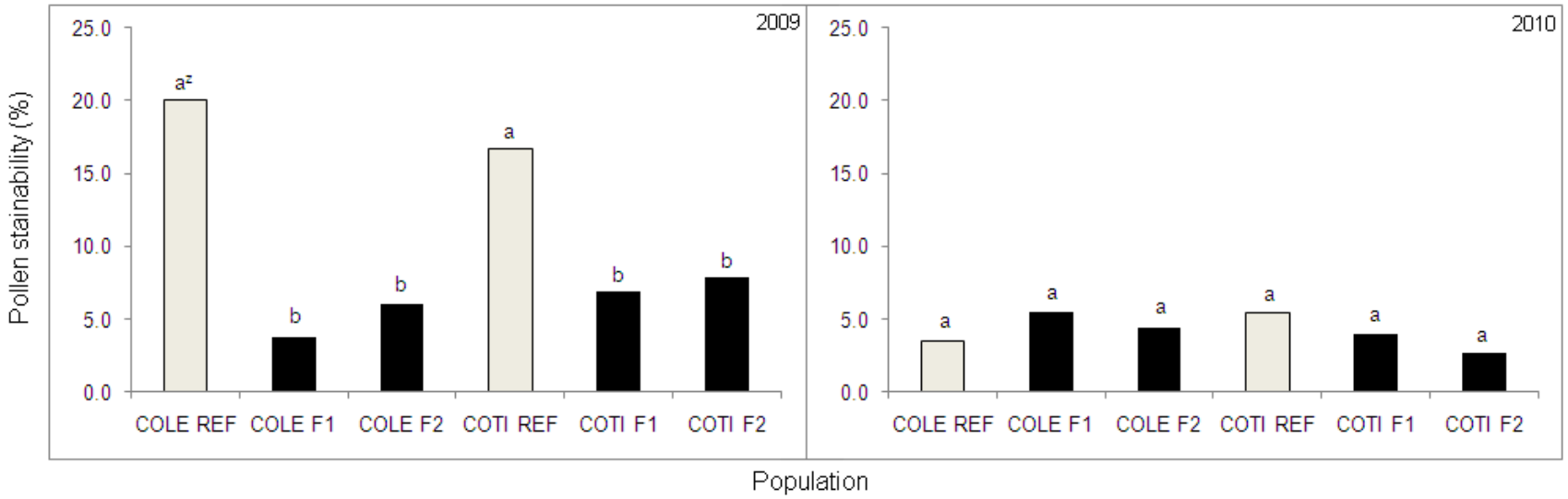

Figure 4-7. Differences in the rate of pollen stainability of six populations of *C. leavenworthii* and *C. tinctoria* produced at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010. [z]Means within year not followed by same letter are significantly different by Tukey's W Procedure at p≤0.05.

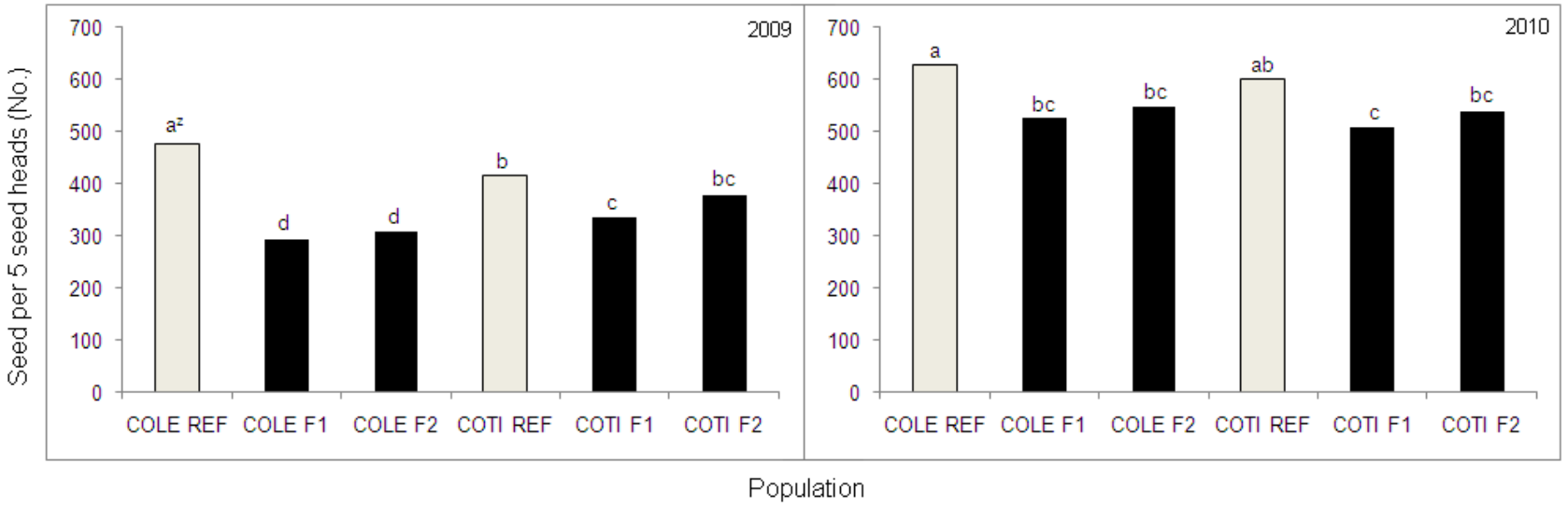

Figure 4-8. Differences in the amount of seed produced from five seed heads in the field of six populations of *C. leavenworthii* and *C. tinctoria* produced at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010. [z]Means within year not followed by same letter are significantly different by Tukey's W Procedure at p≤0.05.

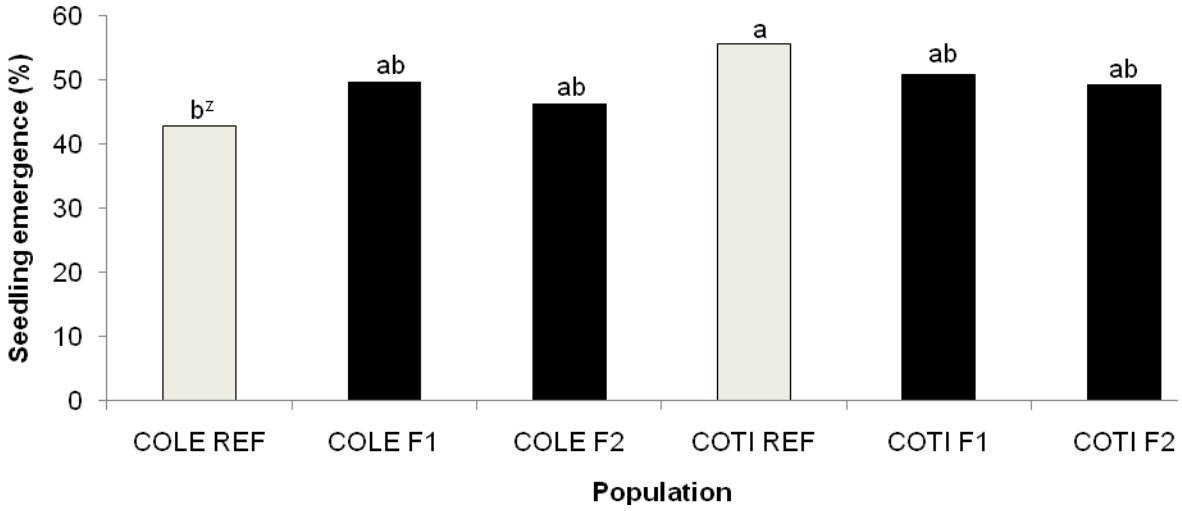

Figure 4-9. Differences in the rate of seed germination of seed collected in the field of six populations of *C. leavenworthii* and *C. tinctoria* produced at the Gulf Coast Research and Education Center, Wimauma, FL in 2009. [z]Means not followed by same letter are significantly different by Tukey's W Procedure at p≤0.05.

CHAPTER 5
POLLEN-MEDIATED GENE FLOW FROM *COREOPSIS TINCTORIA* TO *COREOPSIS LEAVENWORTHII*: IDENTIFYING MORPHOLOGICAL MARKERS AND DETERMINING GENE FLOW RATE AND POLLEN TRAVEL DISTANCE

Justification

When two compatible species are in close proximity and have overlapping flowering periods, interspecific pollen-mediated gene flow can occur (Ellstrand et al., 1999). Many factors can affect gene flow rate, such as plant species and density, fertility, flowering synchrony, number of plants flowering at the same time, vector type, nectar supply, topography, wind speed and direction, temperature and humidity (Bateman, 1947; Beckie and Hall, 2008). The lower the density of plants in a population, the greater the swamping effect can be from gene flow and little gene flow can be evolutionarily significant for a population or species (Antonovics, 1976; Ellstrand, 1992b). A larger pollen source population can cause a greater rate of gene flow (Ellstrand et al., 1989). Because gene flow can be affected by so many variables, the magnitude of gene flow can vary between seasons, years, species and individuals (Ellstrand et al., 1999).

The pollination biology of a species is a major determining factor of pollen-mediated gene flow. Gene flow typically occurs at greater distances for outcrossing species than inbreeding species. A species can be wind, water, insect or bird pollinated, which can greatly affect the rate and distance of gene flow. The genus *Coreopsis* is typically pollinated by insects. For insect pollinated species, gene flow is greatly dependent on the type of pollinator that forages on a crop because it can be a generalist or a specialist and can determine flight distance due to reward and energy requirements (Schmitt, 1980). Bees have been recorded as moving between near-

neighbor plants and move short distances if the rewards are suitable, which could restrict gene flow. Bees typically visit more flower heads per plant and more plants per foraging session compared to other insects.

Bees can use pollen and nectar to feed larvae instead of visiting flowers for nectar collecting purposes that result in pollination of the flower heads (Muller and Kuhlmann, 2008). Plants have evolved various mechanisms to alter the nutritional quality and toxicity of the pollen to limit pollen loss, as found in Asteraceae. In general, insect pollinators forage on plants based on the energy requirements to get to the plants and the energy rewards that the nectar will give the insect (Schmitt, 1980). While different insects have different foraging patterns, insect foraging can have a great affect on gene flow. If the energy and time spent traveling to a population is too costly, the insects will restrict the distance traveled (Osborne et al., 2008). Insects continue feeding on a flower until the nectar of that flower is depleted (Levin, 1981).

Insect pollinated plants typically receive pollen from many plants (Chapman et al., 2005). If two species are growing within the range of vector travel, a plant could receive pollen from either or both species. In some cases pollination with heterospecific pollen has reduced pollen germination on the stigma, slower pollen tube growth in the style and decreased fertilization of the ovules, which can result in fewer interspecific seed forming, compared to pollination with conspecific pollen.

The rate of pollen-mediated gene flow between species can be estimated by growing both species in experimental fields. Two types of experimental arrangements have been commonly used to determine pollen-mediated gene flow between species (Beckie and Hall, 2008). In a continuous experimental design, the pollen trap species is

planted around the centralized pollen donor species. In the discontinuous design, the pollen trap species is several distances apart from the pollen donor species. Spatial arrangements and densities of the pollen donor and trap populations need to be carefully considered as they can have an effect on gene flow rates (Bateman, 1947).

Pollen-mediated gene flow typically follows a leptokurtic distribution, where most gene flow occurs between plants that are closer together and decreases at greater distances with most gene flow occurring within 20 m of the source population (Arriola and Ellstrand, 1996; Ellstrand, 1992a; Levin, 1981). Animal and insect vectors rarely carry pollen more than 1000 m and generally carry pollen farther distances when plant density is low. In many experimental designs, gene flow rates are underestimated at distances greater than 30 m because it is difficult to accurately assess all of the interactions between the individuals, populations and insects carrying pollen (Levin, 1981).

Arriola and Ellstrand (1996) conducted an experiment where interspecific hybridization was assessed by planting *Sorghum halepense* around a field of *S. bicolor* up to 100 m away, which took place at two locations over two years. Pollen-mediated gene flow was greater at distances closer to the pollen source (*S. halepense*) location at about a rate of 10% gene flow at 0 m. At the 5 m distance the gene flow rate decreased to about 3%. Gene flow did occur at the 100 m distance at a rate as high as 2% at one location one year and was highly variable depending on the year and location of the study. *S. bicolor* is an outcrossing species, whereas *S. halepanse* is typically a self pollinated species but will outcross. *S. bicolor* and *S. halepense* are both wind pollinated and were reported to cross pollinate spontaneously in nature, and

interspecific hybridization occurred successfully by hand pollinations. Both species of *Sorghum* grow in the same areas and have overlapping flowering periods.

As in the study with *Sorghum*, *Coreopsis leavenworthii* and *C. tinctoria* have both been reported as growing in similar locations, where both species have been reported as growing in Florida. *C. leavenworthii* has been reported in most counties in Florida and two counties in Alabama and is considered endemic to Florida. *C. tinctoria* has been reported in most of the United States, including some counties in Florida where *C. leavenworthii* also exists. Because it has been reported that *C. leavenworthii* and *C. tinctoria* are cross compatible by Smith (1976) and confirmed by the synthetic F_1 and F_2 populations produced in Chapter 4, it is possible that these two species could cross pollinate when grown at a certain distance from each other. The possible distance and frequency of gene flow is not known. The distance of pollen-mediated gene flow that could occur between these two species needs to be assessed to help prevent natural interspecific hybridization. Previous results from Chapter 4 indicate that the F_1 hybrids are relatively vigorous and are reproductively viable, although there was a decrease in viability in the interspecific F_1 and F_2 hybrids. These results indicate that the interspecific hybrids could compete and intercross with intraspecific populations. The objectives of this study were to determine the inheritance of morphological markers that could potentially be used to reliably detect pollen-mediated gene flow, to assess the effects of planting distance on pollen-mediated gene flow from *C. tinctoria* to *C. leavenworthii* and to identify insect pollinators that may be involved with pollen-mediated gene flow.

Materials and Methods

Seed Source

The *C. leavenworthii* (COLE) plants used in this study were from seed collected by Nancy Bissett from a natural *C. leavenworthii* population at the Reedy Creek Mitigation Bank in Polk County, FL. The *C. tinctoria* (COTI) plants were produced from seed that was purchased from Wildseed Farms in Fredericksburg, TX.

Identifying Morphological Differences between *C. leavenworthii* and *C. tinctoria*

Plants of the two species were grown side by side and closely examined during the course of study to find morphological characteristics that were present in one species but absent in the other species. About one hundred plants were assessed for morphological differences in the flowers, leaves, stems and seed to determine the inheritance pattern of these traits for possible use as a morphological marker to detect pollen-mediated gene flow.

Inheritance of Morphological Traits

Crosses and populations

Five types of crosses were made to investigate the inheritance of three morphological traits (Figure 5-1) in *C. leavenworthii* and *C. tinctoria*, including intraspecific crosses for each species, reciprocal interspecific crosses between the two species, selfing and sibling of interspecific hybrids and testcrosses. Hand pollinations were carried out on plants at the University of Florida, Gulf Coast Research and Education Center, Wimauma, FL. Parental plants for all cross types were grown in 3.2-L plastic containers filled with Vergrow verlite container mix A (Tampa, FL) mixed with 8.6 g of 15N-3.9P-10K (5-6 mo. Osmocote, The Scotts Co., LLC, Marysville, OH) per container in the greenhouse (29.4/23.9°C). The only exception was the parental plants

for the testcross pollinations were grown in 2.1-L containers and the soil media was mixed with 5.6 g of 15N-3.9P-10K per container. For all types of crosses, the flower heads were bagged at the bud stage. Pollen was collected by tapping the whole flower head in a plastic container, where the pollen was deposited. To pollinate flower heads of the maternal plant, they were dipped into the container and rubbed against the available pollen in the container. The pollinated flower head was rebagged and the bag was kept on until the seed head was matured and harvested.

Seed from all crosses were sowed on the surface of Vergrow verlite container mix A in 532-mL containers in the greenhouse (29.4/23.9°C) and kept under mist irrigation. Seedlings were transplanted into 148-mL cell Speedling flats (32-cell flats) (Sun City, FL) of Vergrow verlite container mix A mixed with 0.4 g of 15N-3.9P-10K per cell. The only exception was an initial group of interspecific seedlings were transplanted in 31-mL cell Speedling flats (128-cell flats).

Intraspecific crosses and populations. Synthetic *C. leavenworthii* (COLE) intraspecific crosses were made between 27 July and 5 August 2007 and *C. tinctoria* (COTI) intraspecific crosses were made between 7 and 10 August 2007. Seed were sowed on 11 January 2008. Seedlings were transplanted on 2 February and fertilized with 300 ppm of 15N-13P-12.5K (Plantex, Plant Products CO. Ltd., Brampton Ontario, Canada).

Interspecific crosses and F_1 populations. Four COLE plants (L1 to L4) and four COTI plants (T1 to T4) were randomly chosen and cross-pollinated in 32 combinations between 14 June and 30 July 2007. Sixteen of the crosses had the COLE plants as the maternal parent (COLE x COTI) and sixteen had COTI as the maternal parent (COTI x

COLE). An initial portion of seed from eight of the populations was sowed on 23 October 2007 to begin to assess the inheritance patterns of the traits of the F_1 interspecific hybrids. Seedlings were transplanted on 15 November and fertilized with 20N-2.2P-25K (Peter's Professional, The Scotts Co., LLC, Marysville, OH). The field was a mix of Myakka, Haplaquents, and St. Johns sandy soil. The beds were raised 20 cm high, 61 cm wide at the top and 71 cm wide at the base. The beds were fumigated with 67% methyl bromide: 33% chloropicrin at 197 kg ha^{-1}. The plants were transplanted in the field on 30 and 31 January 2008 and spaced 30.5 cm apart within plots and 122 cm in rows. The plants were fertilized with a total of 153 kg ha^{-1} of nitrogen and potassium throughout the season through drip irrigation. Phosphorus was added throughout the season as needed. To obtain additional trait inheritance data on the remaining interspecific F_1 populations and those already assessed, more interspecific seed were sowed on 27 June 2008. Seedlings were transplanted on 14 July and fertilized with 300 ppm of 20N-8.7P-16.7K (Peter's, The Scott's Co., LLC, Marysville, OH).

F_1 selfing and sibling crosses and F_2 populations. Two types of F_2 populations were produced by 1) selfing (seven populations) or 2) sibling F_1 hybrids (five populations). The seed from selfing and sibling crosses were sowed in August 2008. All seedlings from both types of crosses were transplanted on 21 August and fertilized with 300 ppm of 20N-8.7P-16.7K.

Testcrosses and populations. The testcross populations were produced from 1 July to 6 August 2007, where eight populations were produced. To produce these populations, COLE x COTI interspecific hybrids were backcrossed with COLE. The

seed were sowed on 27 October. Seedlings were transplanted on 18 November and fertilized with 300 ppm of 20N-8.7P-16.7K.

Seed production analysis. Prior to sowing the seed, the number of seed from each cross was counted. Seed production of each cross was statistically analyzed using the ANOVA using PROC GLM and Tukey's W Procedure in SAS (SAS, 1997).

Progeny evaluation

Trichomes. When F_1 progeny were about 80-days old, they were assessed for the presence or absence of trichomes on the leaf petiole.

Seed wings. The seed from the intraspecific COLE, intraspecific COTI, F_1 hybrid and the F_2 sibling crosses were evaluated for the presence or absence of the wingedness of the achene.

Maroon spot. The presence or absence of the maroon spot was recorded for each plant of both intraspecific, F_1, F_2 and testcross populations. The size of the maroon spot was evaluated by measuring three ray petals from three flower heads (cm) for each plant of the 32 F_1 hybrid populations. The measurements from the three flowers per plant were averaged, which were then averaged for each population.

Trait segregation analysis

The inheritance patterns were statistically tested using the chi square goodness of fit test using a one gene model.

Assessing Pollen-mediated Gene Flow

Setting up pollen source and trapping plots

C. leavenworthii plants were planted in the field in plots at 1.5, 3.0, 7.6, 15.2, 30.5, 45.7, 61.0, 76.2 and 91.4 m away from *C. tinctoria* plants during 2007 and 2008 at the University of Florida, Gulf Coast Research and Education Center, Wimauma, FL (Figure

5-2). The plots were planted in a discontinuous design, where *C. leavenworthii* plots were in the same bed but plots were not in contact with each other. For both years, there were three blocks with 16 plants per plot and the blocks were planted at least 91.4 m away from each other. Once the progeny of each plot was grown out, data was recorded on the presence or absence of the maroon spot for each plant as they bloomed. About 5,700 and 5,600 plants were counted for 2007 and 2008, respectively.

2007. *C. leavenworthii* and *C. tinctoria* seed were sowed on 16 January 2007 into 532-mL containers on the surface of Vergrow verlite container mix A media in the greenhouse and put under mist. The seedlings were transplanted about three weeks later in 80-mL cells (Landmark Plastic Corp., Akron, OH) of Vergrow verlite container mix A media. The field was a mix of Myakka, Haplaquents and St. Johns sandy soil. The beds were raised 25 cm high, 71 cm wide at the top and 81 cm wide at the base. The beds were fumigated with 67% methyl bromide: 33% chloropicrin at 197 kg ha^{-1}. Plants were spaced 30.5 cm apart within plots that were 92 cm apart in rows with 152 cm between rows. The plants were transplanted in the field on 20 April, and each bed contained two rows of plants. The plants were fertilized with a total of 169 kg ha^{-1} of nitrogen, 37 kg ha^{-1} of phosphorus and 210 kg ha^{-1} of potassium through drip irrigation.

2008. *C. leavenworthii* and *C. tinctoria* seed were sowed on 9 January 2008 and transplanted into 80-mL cells of Vergrow verlite container mix A media on 13 February. The plants were transplanted into the field on 26 March. All other growing conditions were the same as the previous year.

Collecting seed from pollen trapping plots

Seed heads were harvested from the plants grown in 2007 on 31 May to 4 June, 5 July and 11 to 14 July 2007 and from the plants grown in 2008 on 28 May, 25 June and

17 and 18 July 2008 for harvests 1, 2 and 3, respectively. Plants were in full bloom around the end of April for both years and ten seed heads were harvested per plant for all harvests.

Detecting gene flow events in *C. leavenworthii* progeny

2007. Seed collected from the second and third harvests in 2007 were sowed on 7 September 2007 in a 2.1-L plastic container of Fafard soil mix 1P (Anderson, SC) at the University of Florida, Gainesville, FL and placed under mist. The seedlings were transplanted on 12 October into 80-mL cell trays of Fafard soil mix 2P (Anderson, SC) and fertilized with 150 ppm of 20N-8.7P-16.7K. Resets were completed on 19 and 25 October. Seed collected from the second and third harvests in 2007 were sowed on 10 and 11 January 2008 at the University of Florida, Gulf Coast Research and Education Center, Wimauma, FL. The seedlings were transplanted on 15 and 18 February into 31-mL Speedling trays of Vergrow verlite container mix A. The beds were raised 20 cm high, 61 cm wide at the top and 71 cm wide at the base. The plants were spaced 30.5 cm apart within plots and 122 cm in rows. The beds were fumigated with 67% methyl bromide: 33% chloropicrin at 197 kg ha^{-1}. The plants were transplanted in the field on 2 April. The plants were fertilized with a total of 153 kg ha^{-1} of nitrogen and potassium through drip irrigation. Phosphorus was added as needed. The data for the plants grown in Gainesville, FL and Wimauma, FL was combined for 2007.

2008. Seed collected from the first and second harvests in 2008 were sowed on 26 June 2008 in a 2.1-L plastic container of on the surface of Vergrow verlite container mix A and put under mist. The seedlings were transplanted into 31-mL cell Speedling flats of Vergrow verlite container mix A on 6-8 August and were fertilized with 300 ppm of 20N-8.7P-16.7K. Resets were done on 12, 13, 14, 20 and 27 August. The plants

were planted in the field on 12 and 15 September. All other growing conditions were the same as those at the Gulf Coast Research and Education Center in 2007.

Statistical analysis

For each year, the percentage of plants with the maroon spot present out of the total number of plants evaluated was calculated for each block and then combined. The gene flow rate for each distance and year was fit to a logistic regression curve using PROC GENMOD for the Logistics procedure in SAS (SAS, 1997). The Logistics procedure can be used to analyze binary response, where an individual can take one of two possible values. In the case of the current study, it was used to analyze the maroon spot, where it was either present or absent on the ray flower. Data were fit to the following equation:

$$p=1/(1+e^{3.2387+0.1673D-0.00124D^2})$$

Where p= the probability at a specific distance that gene flow will occur, e=the inverse of the natural logarithm and D=the distance at which gene flow was being measured.

Results

Crossability between *C. leavenworthii* and *C. tinctoria*

On average 46.4 seed per seed head were produced from five *C. leavenworthii* (COLE) maternal individuals that were crossed using the pollen from 40 *C. leavenworthii* individuals (Table 5-1). On average 18.5 seed per seed head were produced from five *C. tinctoria* (COTI) maternal individuals that were crossed using the pollen from 35 individuals. The COLE intraspecific crosses produced 150.8% more seed per seed head than the COTI intraspecific crosses, which is congruent with the

results in Chapter 4, with *C. leavenworthii* having more disk flowers per flower head than *C. tinctoria*.

On average COLE x COTI interspecific crosses produced 53.9 seed per seed head, which was similar to that found in Chapter 4, where 61.9 seed per seed head were produced. COTI x COLE interspecific crosses produced on average 27.7 seed per seed head, which was similar to that found in Chapter 4, where 25.0 seed per seed head were produced, (Table 5-1). The COLE x COTI crosses produced 16.2% more seed than the COLE intraspecific crosses and the COTI x COLE crosses produced 49.7% more seed than the COTI intraspecific crosses. Because both types of F_1 interspecific crosses produced more seed than the intraspecific crosses in the previous (Chapter 4) and current studies, these two species appear to be fully compatible.

On average the F_1 self crosses produced 2.8 seed per seed head and the F_1 sibling crosses produced 17.8 seed per seed head (Table 5-1), which was a 535.7% increase for the F_1 sibling crosses. The F_1 self crosses produced 5.2% and 10.1% of the seed per seed head that the COLE x COTI and COTI x COLE crosses produced, respectively. The F_1 sibling crosses produced 33.0% and 64.3% of the seed per seed head that the COLE x COTI and COTI x COLE crosses produced, respectively. On average the testcrosses produced 18.4 seed per seed head, which was a 3.4% increase from the F_1 sibling and a 557.1% increase from the F_1 selfing crosses in seed per seed heads. The testcrosses produced 34.1% and 66.4% of the seed per seed head that the COLE x COTI and COTI x COLE crosses produced, respectively. Because there was a reduction in seed produced per seed head for both types of F_1 crosses and the testcrosses, hybrid breakdown appears to be occurring.

Morphological Differences between *C. leavenworthii* and *C. tinctoria*

Three morphological character differences were evaluated, which were trichomes, seed wingedness and the maroon spot on ray flowers (Figure 5-1). Trichomes were found to be absent in 86 *C. leavenworthii* plants and present in 35 *C. tinctoria* plants. Seed wingedness was found to be present in 5722 *C. leavenworthii* seed but absent in 870 *C. tinctoria* seed. The maroon spot was found to be absent in 86 *C. leavenworthii* plants and present in 35 *C. tinctoria* plants. Intra- and interspecific crosses were made between the two species to evaluate the inheritance patterns for these characteristics.

Expression and Inheritance of Trichomes

Trichomes were present on all F_1 hybrid plants (about 1000 plants evaluated), COLE x COTI and COTI x COLE, in a 1:0 genetic ratio, indicating that trichomes were dominantly inherited from *C. tinctoria*. However, the size of trichomes and number varied among F_1 plants but the size and number were consistent within each plant. The trichomes were 1 or 2 per leaf petiole to as many as 20 per leaf petiole and could be long (up to 5 mm) or short (about 2 mm) in size. All combinations were found on the F_1 hybrid plants. When interspecific hybrids were assessed for trichomes in a previous study at the North Florida Research Education Center (Quincy, FL), 38 interspecific hybrids did not have any trichomes visible to the naked eye, 21 had few, visible trichomes and 21 had numerous, visible trichomes (personal communication). This pattern of segregation suggests that the presence of trichomes on *C. tinctoria* leaf petioles might be controlled by more than one locus and the loci were not in homozygosity. Because of this nature, trichomes would not be the ideal morphological marker for tracking pollen-mediated gene flow from *C. tinctoria* to *C. leavenworthii*.

Expression and Inheritance of Maroon Spots

Presence of maroon spots

Intraspecific crosses from five COLE seed parents using 40 COLE pollen plants resulted in 86 progeny, and they all lacked the maroon spot. Intraspecific crosses from five COTI seed parents were made using 35 COTI pollen plants. Thirty-five progeny were produced that all expressed the maroon spot on the ray flowers (Table 5-2). Sixteen COTI x COLE crosses using four COLE and four COTI plants produced 493 progeny and all expressed the maroon spot, indicating that the presence of the maroon spot is dominant over no spot. The maroon spot was expressed in an additional 525 progeny produced from reciprocal COLE x COTI crosses, suggesting that the trait is under nuclear gene control.

Selfing F_1 plants produced only a limited number of F_2 progeny (32 individuals). Of them, 25 expressed the maroon spot and seven lacked the maroon spot, thus the presence of the maroon spots segregated into a 3:1 ratio (χ^2=0-1.33 and *P*=0.25-1.00) as expected for a single dominant gene-controlled trait. Sibling F_1 plants produced more progeny (306). Among these F_2 progeny 227 expressed the maroon spot and 79 plants did not. Again the trait segregated into a 3:1 ratio (χ^2=0.005-1.45 and *P*=0.23-0.94). When F_1 interspecific (COLE x COTI) individuals were backcrossed with COLE, 163 out of 370 progeny expressed the maroon spot and 207 did not. The segregation between the presence and absence of the maroon spot fit a 1:1 ratio (χ^2=0.12-2.60 and *P*=0.11-0.73). These results show that the presence of the maroon spot was controlled by a single dominant homozygous nuclear gene in *C. tinctoria* and was easily identified in the interspecific hybrids making it a reliable morphological marker to identify pollen-mediated gene flow from *C. tinctoria* to *C. leavenworthii*.

Size of maroon spots

This size of the maroon spot for *C. tinctoria* individuals (T1-T4) was larger for T2 and T3 and smaller for T1 and T4. These individuals were used as the maternal parents in interspecific crosses (COTI x COLE) and they produced 384 progeny. When T1, T2, T3 and T4 were used as the maternal parent, the average maroon spot size of the progeny was 0.43 (120 individuals), 0.50 (103 individuals), 0.55 (80 individuals) and 0.21 (81 individuals) cm, respectively (Figure 5-3). When *C. tinctoria* was used as the maternal parent and crossed with any of the four *C. leavenworthii* parents, the size of the maroon spot of the progeny was largest for T3, T2, T1 and T4, respectively.

To determine if there was a maternal effect on the size of the maroon spot, reciprocal crosses were made with *C. leavenworthii* (L1-L4) as the maternal parent and *C. tinctoria* as the paternal parent (COLE x COTI), and these crosses produced 355 progeny. When T1, T2, T3 and T4 were used as the paternal parent, the average maroon spot size of the progeny was 0.41 (102 individuals), 0.49 (90 individuals), 0.63 (89 individuals) and 0.19 (74 individuals) cm, respectively (Figure 5-3). When *C. tinctoria* was used as the paternal parent, independent of the *C. leavenworthii* maternal parent, the size of the maroon spot of the progeny was largest for T3, T2, T1 and T4, respectively. Because the same pattern was observed when *C. tinctoria* was used as the maternal and paternal parent, there is not a maternal or reciprocal effect and the maroon spot size was solely dependent on the maroon spot size of the *C. tinctoria* parent.

Expression and Inheritance of Seed Wings

All COLE intraspecific crosses produced winged seed (5722 seed evaluated) and all COTI intraspecific crosses produced non-winged seed (807 seed evaluated) (Table

5-3). When COLE plants were used as the seed parents and crossed with COTI pollen (COLE x COTI), all of the seed (7433) were winged. When COTI plants were used as the seed parents and crossed with COLE pollen (COTI x COLE), all seed were non-winged for 26 of the crosses. However, the trait segregated in four other COTI x COLE crosses, 52 winged seed: 89 non-winged seed. When interspecific F_1 plants (COLE x COTI) were self pollinated, two crosses produced winged seed (23), three crosses produced winged seed (48) and non-winged seed (375) and one cross produced only non-winged seed (100). The segregation of seed wingedness was possibly due to the phase of seed development that the *C. tinctoria* plants were undergoing when the crosses were completed or the seed source (Parker, 1973). *C. tinctoria* has a winged and wingless phase, and segregation in seed wingedness in *C. leavenworthii* and *C. tinctoria* interspecific hybrids was found as well, indicating that the inheritance of seed wingedness was incompletely dominant because it was not completely dependent on the winged phenotype of the *C. tinctoria* parent.

Pollen-mediated Gene Flow from *C. tinctoria* to *C. leavenworthii*

For each pollen trap plot, 581-681 progeny were evaluated in 2007 and another 593-654 progeny were evaluated in 2008 to identify the progeny from the *C. leavenworthii* individuals expressing *C. tinctoria*'s maroon spots. According to the above-described inheritance study, these individuals would indicate pollen-mediated gene flow from *C. tinctoria* into *C. leavenworthii*. The identified individuals were then used to calculate gene flow rates.

There was no significant difference in gene flow rate among blocks in 2007, but in 2008 the gene flow rate in Block 3 seemed to be much lower than in Blocks 1 and 2, and these blocks were thus evaluated separately. For both years the 1.5 m plot had the

highest rate of gene flow, 3.89% (25 out of 646 individuals) in 2007 (averaged from three blocks), 4.24% (17 out of 401 individuals) in 2008 for Blocks 1 and 2 and 0.93% (2 out of 213 individuals) in 2008 for Block 3 (Figure 5-4). In 2007 and 2008 the farthest distance that gene flow occurred was 61.0 m (1 out of 649 individuals) and 15.2 m (1 out of 430 individuals), respectively. At these distances, the observed gene flow rates were 0.13% and 0.23%, respectively.

ANOVA results indicated that the gene flow rates were not significantly different between the two years (F=1.85, *P*=0.1829), so the gene flow rate data from the two years were pooled and a regression curve was fit to the observed data using the Logistics procedure (Table 5-4). The curve shows that the closer the plants were grown, the more likely gene flow can occur, where $p=1/(1+e^{3.2387+0.1673D-0.00124D^2})$ (Figure 5-5). The equation was used to estimate the probability of gene flow events at a particular distance. Based on this equation, separating the species by 28 m or 60 m would lower the gene flow rate to about 0.10% or 0.01%, respectively.

Discussion

Crossability of *C. leavenworthii* and *C. tinctoria*

Based on the amount of seed produced from the interspecific crosses, the two species were compatible and could produce a large number of seed when hand-pollinated, which was congruent with the results in Chapter 4 and a previous report by Smith (1976). The amount of seed per seed head produced by the COLE x COTI crosses (53.9 seed per seed head) was similar to that by COLE intraspecific crosses (46.4 seed per seed head). The COTI x COLE crosses produced 27.7 seed per seed head, similar to that of COTI intraspecific crosses (18.5 seed per seed head). The amount of seed produced per seed head by the COLE x COTI and COTI x COLE

crosses in the current study was similar to the results from the previous study (61.9 seed per seed head for COLE x COTI crosses and 25.0 seed per seed head for COTI x COLE crosses in Chapter 4). In both studies, more seed were produced per seed head when COLE was the maternal parent than when COTI was the maternal parent.

The F_1 sibling and testcrosses produced an amount of seed per seed head comparable to the COTI intraspecific pollinations. The F_1 sibling crosses produced an average of 17.8 seed per seed head and the testcrosses produced an average of 18.4 seed per seed head, which were lower than the mid-parent value (32.5 seed per seed head). Fewer seed were produced from the F_1 sibling crosses compared to the COLE x COTI and COTI x COLE crosses, which was likely due to outbreeding depression.

The F_1 individuals continued to produce seed when cross-pollinated with other F_1 plants. The F_1 sibling crosses produced 17.8 seed per seed head, which was similar to that found in the previous study (Chapter 4), where 35.3 seed per seed head were produced. This suggests that the interspecific hybrids and later generations would continue to outcross, which would allow the interspecific plants to continue spreading its pollen to either parental species or interspecific plants. Outcrossing species can be at a greater risk of gene flow and possible extinction (Ellstrand, 1992b).

The F_1 self crosses produced only about three seed per seed head, much fewer than any other type of cross in this study (Table 5-1), illustrating strong self incompatibility in these *C. leavenworthii-C. tinctoria* hybrids. The genus *Coreopsis*, as well as Asteraceae, is known to be sporophytically self-incompatible (Brewbaker, 1957). A self incompatibility study with *C. tinctoria* had suggested that there is a minimum of seven alleles controlling sporophytic self incompatibility (Sharma, 1971). Two other

Coreopsis species, *C. californica* and *C. bigelovii*, in this same study were found to have at least five alleles controlling the sporophytic self incompatibility mechanism. The self incompatibility of the F_1 self crosses showed that these alleles were inherited in interspecific crosses and would promote outcrossing, which could result in more outcrossing between interspecific hybrids and backcrossing with *C. leavenworthii* and *C. tinctoria*.

Trichomes and Seed Wings were not Suitable for Detecting Gene Flow Events

Presence of trichomes in *C. tinctoria* appeared to be a dominant trait, as in the F_1 interspecific hybrids. But the expression of this trait varied considerably among progeny. Further, it appeared to be segregating in the F_1 progeny of *C. tinctoria* and *C. leavenworthii* (Jeff Norcini, personal communication), but could also be due to the lack of visibility without the use of magnification. These results suggest that the trait may be controlled by two or more loci and some of the loci may be heterozygous. Thus, this trait may allow detection of some pollen-mediated gene flow from *C. tinctoria* into *C. leavenworthii*, but it is very likely that the gene flow rates based on such a marker will be underestimated and inaccurate.

The wingedness of the achene was not a reliable marker because it did not follow the inheritance pattern of a homozygous dominantly controlled gene in the F_1 or F_2 generations. Smith (1983) reported *C. tinctoria* seed ranging in seed wingedness from absent to fully winged. When *C. tinctoria* and *C. cardaminefolia* were cross-pollinated, all of the F_1 progeny produced seed with partial wings and the F_2 progeny produced seed ranging from non-winged to fully winged (Smith and Parker, 1971). It was concluded that seed wingedness was dominantly controlled by one or two loci. Parker (1973) reported that *C. tinctoria* had a winged and wingless phase and differed in

wingedness depending on the source of the seed, which determined the wingedness of the seed produced. The wingedness of *C. tinctoria* was found to be incompletely dominant by Parker (1973), which was further supported when crossed with *C. leavenworthii* because some seed were winged and others non-winged. Because of the range in seed wingedness of *C. tinctoria* reported, different ecotypes of this species may have different genotypes for seed wingedness, making it an unreliable morphological marker for gene flow detection.

Presence of Maroon Spots is Controlled by a Single Dominant Gene and is an Applicable, Reliable Morphological Marker for Detecting Gene Flow Events

The maroon spot of *C. tinctoria* segregated as a single, dominant gene in all of the populations examined. Additionally, the character was observed in all 1018 plants used for inheritance and gene flow studies. Should the next *C. tinctoria* individual appear non-spotted, the frequency of this recessive phenotype would be 0.000981 (one out of 1019 F_1 individuals), the recessive allele (non-spotted) would occur at a frequency of 0.031327, and the dominant allele (spotted) would be present at a frequency of 0.968673. Thus in case this scenario does occur, the maroon spot will allow the detection of nearly 97% of pollen-mediate gene flow from *C. tinctoria* into *C. leavenworthii*.

Previously, Smith and Parker (1971) crossed *C. tinctoria* with *C. cardaminefolia* (non-spotted) and observed that the maroon spot was expressed in all F_1 progeny and segregated among the F_2 progeny. Two individuals lacked the maroon spot and 25 individuals expressed the maroon spot. The authors were not able to determine the inheritance of the maroon spot based on the available data. It seems that the reported segregation does not fit a 3:1 ratio expected for a single dominant gene-controlled trait

(χ^2=4.46, *P*=0.03). It will remain to be determined whether this deviation is due to small size of the F_2 population or the differences between *C. cardaminefolia* and *C. leavenworthii*.

Size of Maroon Spot is Controlled by Additional Genes

Based on the consistency of the results of the size of the maroon spot of the *C. leavenworthii-C. tinctoria* hybrids, the size of the maroon spot appeared to be dependent on the size of the spot of the *C. tinctoria* parent and to be controlled by multiple alleles with no reciprocal effects (Figure 5-3). Smith and Parker (1971) observed variation in the maroon spot size among progeny of controlled crosses between *C. tinctoria* and *C. cardaminefolia* and hypothesized that the spot size was controlled by two or more loci with additive effects.

Pollen-mediated Gene Flow Rate from *C. tinctoria* to *C. leavenworthii* was Lower than Expected

Because of the high level of crossability found between *C. leavenworthii* and *C. tinctoria* observed in hand pollinations in the greenhouse, it was expected that there would be a relatively high frequency of pollen-mediated gene flow from *C. tinctoria* to *C. leavenworthii* in the field as insects foraged on *Coreopsis* flowers. Plants of both species produced large numbers of flowers in the field, and their flowering time overlapped throughout the year, thus there should be plenty of opportunity for *C. tinctoria* to cross-pollinate *C. leavenworthii*. Nevertheless, the highest rate of pollen-mediated gene flow from *C. tinctoria* into *C. leavenworthii* observed in two years was 4.24% when plants of the two species were planted 1.5 m apart. No gene flow events were detected when plants of the two species were planted 76.2 m and 91.4 m apart.

Arias and Rieseberg (1994) reported that crop and weed hybridization of sunflower occurred at a frequency of gene flow of 0.27 at 3 m and 0.02 at 1000 m. Although these frequencies are much lower than those found in the current study, they can still be biologically significant. From a statistical standpoint the amount of gene flow that could occur in sunflower and in the current study with *Coreopsis*, there was a low chance that an insect would carry pollen from one species to another. From a biological standpoint, a few pollinations between the species could result in a large amount of interspecific seed produced (Grant, 1981). Based on the interspecific seed produced by hand in this study, about 30-50 seed per seed head could be produced from each *C. leavenworthii-C. tinctoria* interspecific pollination, which could turn into many interspecific plants in a natural setting. The vigor of the interspecific hybrids and later generations should be assessed in a mixed population in a natural setting.

Buffer Zones can Protect *C. leavenworthii* from Genetic Contamination

The observed gene flow from *C. tinctoria* to *C. leavenworthii* follows a leptokurtic curve, where the greatest frequency of gene flow occurred at the 1.5 m plot and the gene flow rate consecutively decreased for each plot afterwards. The farthest distance that gene flow occurred was 61.0 m with no gene flow occurring at the 76.2 and 91.4 m plots. The highest frequency of gene flow occurs when the two species are planted closest together typically within a few meters of the pollen donor population (Beckie and Hall, 2008; Ellstrand, 1992b; Levin, 1981). By growing the species farther apart, gene flow will be less likely. Even though most contamination can occur at closer distances, gene flow occurring at farther distances can affect a population also. It only takes one individual per generation to be evolutionarily important. Therefore, the tail end of the leptokurtic tail should not be disregarded (Ellstrand, 1992b). Most gene flow occurred

from the 1.5-15.2 m plots in both years, but some gene flow did occur at the 30.5-61.0 m plots in 2007. Based on these results and the regression curve fit for the data, these two species should be separated by about 60 m to minimize the possibility of gene flow. Arias and Rieseberg (1994) reported that crop and weed hybridization of sunflower occurred from 3-1000 m, suggesting that gene flow can occur at great distances and should be considered when creating buffer zones between two species.

A trap crop may be also utilized to protect *C. leavenworthii*. This entails growing a narrow strip of plants at the edge of the pollen trap field and removing these plants before seed harvest. A pollen trap crop can be the same species as the pollen receptor crop. The rows of the pollen trap crop are expected to "consume" the pollen load from the other species, thus protecting the natural or seed production populations.

The Need to Consider Seed Dispersal on Gene Flow in *Coreopsis*

In addition to pollen-mediated gene flow, seed dispersal may be another important pathway leading to gene flow in *Coreopsis*. This seems especially true considering that a number of *Coreopsis* species, including *C. leavenworthii*, have winged or spiked achenes that can be carried far in gusts of wind or storms (Tadesse et al., 1995). Thus, the possibility of long range seed dispersal exists in *C. leavenworthii* and other *Coreopsis* species (Cain et al., 2000; Levin, 1981; Rukini, 2008). As shown in this and previous studies (Chapter 4 and Smith, 1976, 1983), when *C. leavenworthii* and *C. tinctoria* grow in close proximity, they can cross-pollinate and produce viable hybrids. These hybrids can become a rich source of pollen and seed for further gene flow, interspecific hybridization, genetic swamping and gene pool contamination.

Hymenoptera on *Coreopsis*

Hymenoptera has been reported as pollinating members of the Asteraceae, but specific insects have not been reported as pollinating *Coreopsis* (Arias and Rieseberg, 1994; Levin and Kerster, 1969; Muller and Kuhlmann, 2008). Insects were collected from *C. tinctoria* and *C. leavenworthii* plants on 9 June and 25 September 2008 and identified by the Florida Department of Agriculture and Consumer Services, Division of Plant Industry in Gainesville, FL. For both collections, all of the insects collected were Hymenoptera (Table 5-5). Six Hymenoptera species were found visiting *C. leavenworthii* and four species visiting *C. tinctoria*. Three species, *Halictus poeyi*, *Scolia nobilitata* and *Philanthus ventilabris*, were visiting both species of *Coreopsis*. *Halictus poeyi* and *Scolia nobilatata* are known to be plant pollinators (Triplehorn and Johnson, 2005). It remains to be known whether the other Hymenoptera species pollinate *Coreopsis* plants. It has been reported that some Hymenoptera species collect pollen to feed larvae or look for other insects for feeding. These kinds of insects are generally not hairy for carrying pollen. Some members of Asteraceae have been shown to contain toxic compounds, have low protein content, lack essential nutrients and have pollen grains that are difficult to degrade by insects (Muller and Kuhlmann, 2008). This has caused some members of Hymenoptera to become specialized to members of Asteraceae that are able to overcome these barriers to use pollen for food.

In general larger insects travel farther distances than smaller insects to forage (Greenleaf et al., 2007). Pollinators respond to different stimuli in the environment, and environmental conditions can alter pollinators' foraging patterns (Levin and Kerster, 1969). It has been documented that once insects find a food source, they remain loyal to it and forage over short distances. When the nectar is depleted from one population,

the insects, especially bees, will find the closest population of the same species to forage. Because the nectar rewards of Asteraceae are small, plants within a population must be close in proximity and pollen dispersal will usually remain within that population because bees can probe many florets quickly and maintain a fairly high rate of energy intake (Price, 1997; Schmitt, 1980). In the field the insects seemed to work flowers in a continuous manner (personal observation). They visited a flower and then found another flower in close proximity to visit next. Usually the insects, depending on the species, did not move to a flower that was located several meters away.

The types of crops surrounding the *Coreopsis* plants could explain some of the differences in frequency of gene flow between these two *Coreopsis* species. The *Coreopsis* plants were surrounded by many different vegetable crops and ornamentals in 2007, and they did not seem to affect the rate of gene flow between each block. In 2008, the *Coreopsis* plants were surrounded mostly by ornamentals (Blocks 1 and 2), such as caladium (*Caladium* ×*hortulanum*) and *Lantana camera*. The plants of Block 3 were next to a tomato field (*Solanum lycopersicum*), where there was a much lower rate of gene flow in 2008. Because the gene flow rates were similar between 2007 and Blocks 1 and 2 of 2008, it does not appear that the crops surrounding the *Coreopsis* plants affected the gene flow rates, but because the gene flow rates were much lower in Block 3 of 2008, it is possible that the insects preferred tomato over *Coreopsis*. The greater diversity of crops may have encouraged the greater distances that gene flow occurred in 2007.

Summary

Hand pollination results showed that *C. leavenworthii* and *C. tinctoria* were highly compatible and their interspecific crosses produced similar numbers of seed as their

intraspecific crosses did. Inheritance studies revealed that the maroon spot in *C. tinctoria* was controlled by a single dominant gene and *C. tinctoria* was homozygous at this locus, thus this spot could serve as a reliable morphological marker to reveal gene flow events from *C. tinctoria* to *C. leavenworthii* and quantify the rate of such directional gene flow. Following a discontinuous design, field gene flow studies were conducted in replicated blocks and repeated over two years. Results showed that pollen-mediated gene flow occurred at the highest frequency when the two species were planted 1.5 m apart and continuously occurred as far as the two species were 61.0 m apart. Natural gene flow from *C. tinctoria* to *C. leavenworthii* followed a leptokurtic curve. If validated, this leptokurtic equation will provide a very valuable model for estimating the potential gene flow from *C. tinctoria* to *C. leavenworthii* and planning buffer zones for minimizing such gene flow. Hymenoptera was identified as the main insect pollinators of *Coreopsis*.

Table 5-1. Number of seed collected per seed head for each cross type made at the Gulf Coast Research and Education Center, Wimauma, FL.

Cross type	Seed parent	Pollen parent	Seed head examined (no.)	Seed examined (no.)	Mean
Intraspecific	COLE	COLE	25	696	46.4ab[y]
Intraspecific	COTI	COTI	25	278	18.5cd
Interspecific	COLE	COTI	48	2586	53.9a
Interspecific	COTI	COLE	48	1328	27.7bc
F_1 self	F_1[z]	F_1	21	59	2.8d
F_1 sibling	F_1	F_1	18	321	17.8cd
Testcross	F_1	COLE	24	424	18.4cd

[y] Significant differences determined by Tukey's W Procedure at p≤0.05 with Cross type being significantly different with an F value of 9.91 and p≤0.0001.
[z] F_1 interspecific hybrids were produce by crossing COLE x COTI.

Table 5-2. Inheritance of the maroon spot for *C. leavenworthii* and *C. tinctoria* self, F_1, F_2 and testcross populations.

Cross type	Seed parent	Pollen Parent	Crosses (no.)	Spotted	Non-spotted	Expected Ratio	χ^2 value	*P* value
Intraspecific	COLE	COLE	5	0	86	0:1	0	1
Intraspecific	COTI	COTI	5	35	0	1:0	0	1
Interspecific F_1	COLE	COTI	16	525	0	1:0	0	1
Interspecific F_1	COTI	COLE	16	493	0	1:0	0	1
Interspecific F_2 self	F_1[z]	F_1	7	25	7	3:1	0-1.33	0.25-1.00
Interspecific F_2 sib	F_1	F_1	5	227	79	3:1	0.005-1.45	0.23-0.94
Testcross	F_1	COLE	8	163	207	1:1	0.12-2.60	0.11-0.73

[z]F_1 interspecific hybrids were produce by crossing COLE x COTI.

Table 5-3. Inheritance of seed wingedness in crosses with *C. leavenworthii* and *C. tinctoria*.

Cross type	Seed parent[y]	Pollen parent	Crosses (no.)	Winged	Wingless
Intraspecific	COLE	COLE	40	5722	0
Intraspecific	COTI	COTI	30	0	870
Interspecific	COLE	COTI	40	7433	0
Interspecific	COTI	COLE	26	0	1989
			4	52	89
Interspecific F_1	F_1[z]	F_1	2	23	0
			3	48	375
			1	0	100

[y] Seed counted for this study was collected from the seed parent to produce seed for cross type listed.
[z] F_1 interspecific hybrids were produce by crossing COLE x COTI.

Table 5-4. Chi-square table to determine the probability of gene flow at a specific distance.

Parameter	DF	Estimate	Standard error	Wald Chi Square
Intercept	1	-3.2387	0.1569	426.1609***
Distance	1	-0.1673	0.0295	32.2638***
Distance*Distance	1	0.00124	0.000398	9.7724**

Table 5-5. Hymenoptera found in the field on *C. leavenworthii* and *C. tinctoria* plants at Gulf Coast Research and Education Center, Wimauma, FL.

Family	Genus	Species	Common Name	*C. leavenworthii*	*C. tinctoria*
Colletiae	*Colletes*	sp.	Plasterer bee	1	
Vespidae	*Euodynerus*	sp.	Potter wasp	1	
Halictidae	*Halictus*	*poeyi*	Halictid bee	6	12
Scoliidae	*Scolia*	*nobilitata*	Scoliid wasp	2	1
Sphecidae	*Philanthus*	*ventilabris*	Digger wasp	1	2
Tiphiidae	*Myzinum*	sp.	Tiphiid wasp		2
Vespidae	*Polistes*	*bahamensis*	Paper wasp	1	
Insect species (no.)				6	4

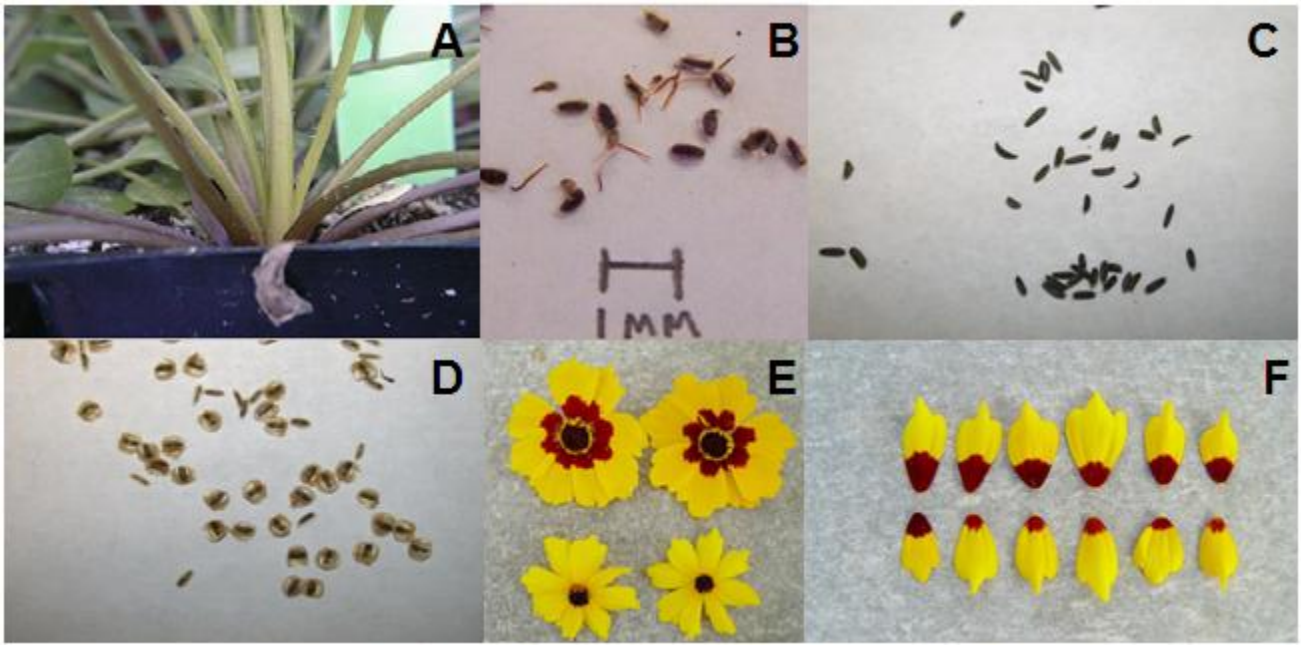

Figure 5-1. Morphological characteristics assessed for potential use as a morphological marker to detect pollen-mediated gene flow. A) Trichomes on the leaf petiole of *C. tinctoria*, B) Seed wings on *C. leavenworthii* seed, C) Non-winged seed of *C. tinctoria*, D) Differences in seed wingedness of COLE x COTI seed, E) Maroon spotted flower of *C. tinctoria* (top) and non-spotted flower of *C. leavenworthii* (bottom) and F) Differences in maroon spot size of *C. tinctoria*.

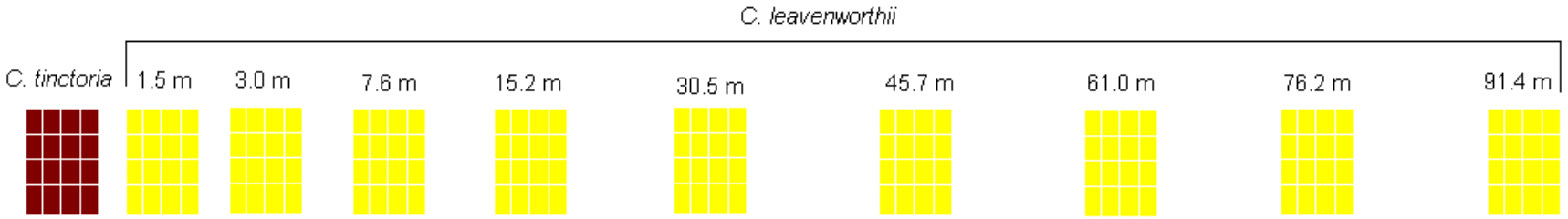

Figure 5-2. Field design of one block out of three for the pollen-mediate gene flow study.

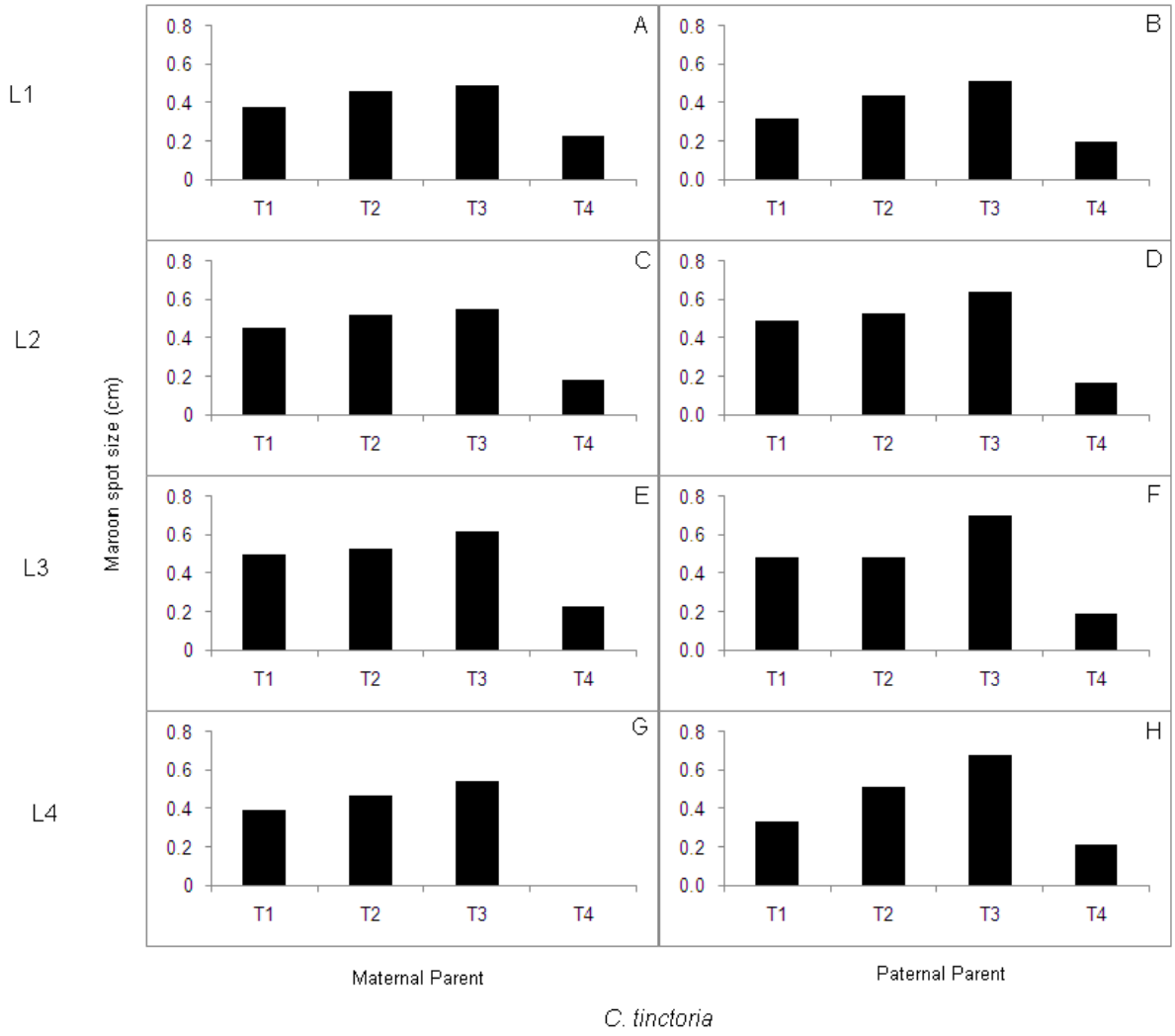

Figure 5-3. The inheritance of the size of the maroon spot in F_1 hybrids from reciprocal crosses between four *C. leavenworthii* and four *C. tinctoria* parents. Figures on the left side indicated the maroon spot size when *C. tinctoria* was used as the maternal parent and those on the right side are when *C. tinctoria* was used as the paternal parent.

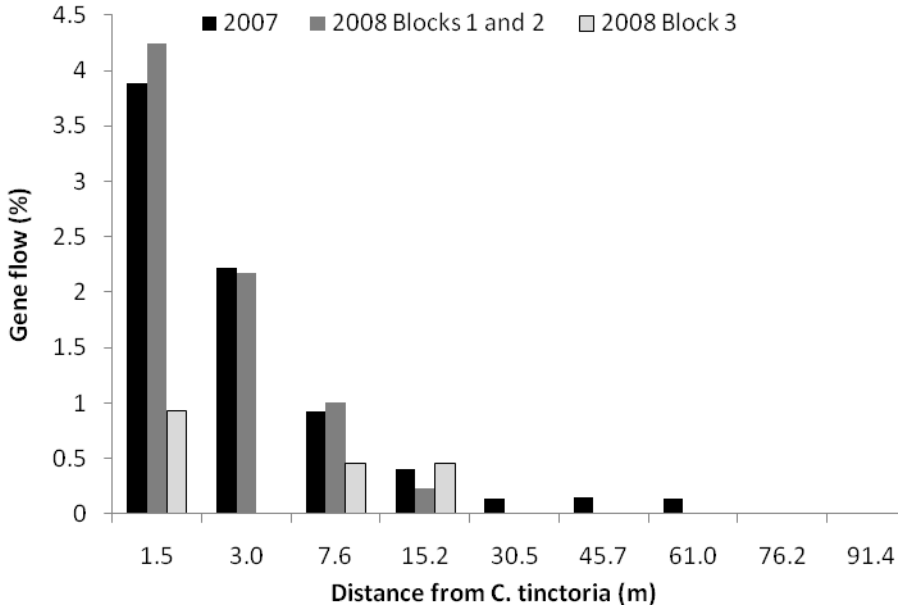

Figure 5-4. Gene flow rates occurring from *C. tinctoria* to *C. leavenworthii* plants at multiple distances in 2007 and 2008 at the Gulf Coast Research and Education Center, Wimauma, FL.

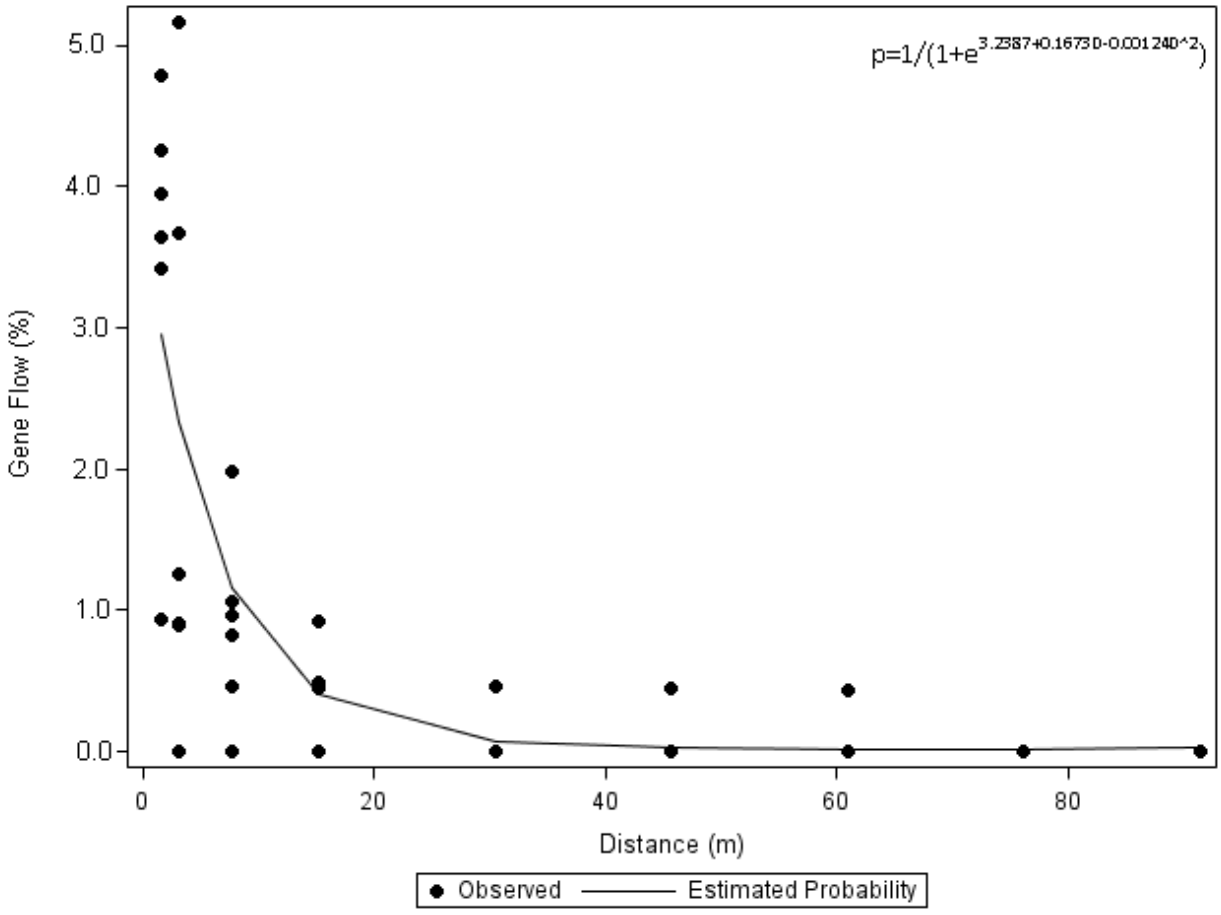

Figure 5-5. Scatter plots of observed gene flow rates at each distance measured and logistic regression curve for the equation fitted to the rate of gene flow from *C. tinctoria* to *C. leavenworthii* at each distance over two years.

CHAPTER 6
CONCLUSIONS

Coreopsis is Florida's state wildflower, and *C. leavenworthii* is one of the 13 species of *Coreopsis* found in Florida. *C. leavenworthii* has been reported as growing in most counties in Florida and only two counties in Alabama. The Florida Department of Transportation (FDOT) grows *C. leavenworthii* for highway beautification and erosion control along roadsides and ditches. Wildflower growers have collected seed from *C. leavenworthii* natural populations and increased seed to meet the demand. Concerns have been raised about the possibility that genetic shift and erosion may occur during seed increase. *C. tinctoria* has been reported as growing in most of the United States, including some counties in Florida but is not native to Florida. It has been reported that these two species are cross-compatible and can produce viable interspecific hybrids. The genetic diversity of *C. leavenworthii* had not been assessed at the phenotypic or molecular levels when seed is increased over successive generations or multiple locations. The effects of interspecific hybridization on the fitness of *C. leavenworthii-C. tinctoria* hybrids were not known. Because both species are growing in Florida, information on the distance and frequency of gene flow was needed.

There were no significant differences between the seed increase populations and the original population (G_0) for the means of most traits evaluated, including plant height, plant dry weight, leaf type, number of days to flower, disk flower size, whole flower size, petal lobing, number of ray petals, seed germination and powdery mildew severity. The mean values for two traits, petal overlap and number of seed produced per five seed heads, did show significant differences between increase populations and G_0, but did not show a consistent trend that indicated a genetic shift. It was concluded

that seed increase over three generations in two climatic zones did not seem to cause any genetic shift or loss of genetic variation in vegetative, physiological and reproductive characteristics of *C. leavenworthii*.

SSR markers were highly polymorphic in *C. leavenworthii* and detected an average of 10.4 alleles per primer pair in G_0. SSR marker analysis revealed subtle changes in alleles and allele frequencies during seed increase. Some of the changes seemed to be due to random genetic drift, as some alleles were present in G_0 but not in the increase populations and some others were not in G_0 but in the increase populations. The total genetic diversity was 0.1736 in G_0 and it slightly decreased in the six increase populations, with the lowest values in the G_3 populations (0.1541 to 0.1706). Compared with G_0, genetic differentiation (G_{ST}) and genetic distance increased slightly as seed increase advanced to later generations (0.0513 and 0.0196, respectively). PCoA did not reveal any distinct clustering of G_0 and the increase populations, suggesting that although there were some genetic changes occurring in the seed increase populations, they were not large enough to cause a significant genetic shift. Therefore, the genetic diversity and integrity of the original populations were maintained during seed increase.

Based on these results, the current seed production practices seem to be appropriate for *C. leavenworthii* and increasing seed in northern or central Florida for three generations did not seem to cause any significant negative effects on *C. leavenworthii* genetic diversity and integrity. Nevertheless, subtle allele and allele frequency changes did occur at the molecular level. Should seed increase be advanced to later generations, it will be important to monitor these changes at phenotypic and

molecular levels and to ensure that *C. leavenworthii*'s genetic diversity and integrity are upheld.

Interspecific crosses between *C. leavenworthii* and *C. tinctoria* produced more seed than their respective intraspecific crosses and interspecific seed germinated well, indicating that *C. leavenworthii* and *C. tinctoria* are fully compatible. Number of days to flower appeared to have heterotic effects in the *C. leavenworthii-C. tinctoria* hybrids. Plant dry weight appeared to be affected by heterosis in the F_1 generation and hybrid breakdown in the F_2 generation. There were significant decreases in the F_1 and F_2 generations for seed production and pollen stainability compared to the intraspecific populations. Pollen stainability was likely affected by chromosome mispairing, while seed production was likely affected by chromosome mispairing and dilution.

Inheritance studies indicated that the maroon spot is controlled by a single dominant gene that is homozygous in *C. tinctoria*, making it a reliable morphological marker for detecting and quantifying pollen-mediated gene flow from *C. tinctoria* to *C. leavenworthii*. The highest observed rate of pollen-mediated gene flow from *C. tinctoria* to *C. leavenworthii* was 4.24% when plants of the two species were grown 1.5 m away. The farthest distance that gene flow was observed was 61.0 m. Several Hymenoptera insects were found on both species of *Coreopsis*, but only two are likely pollinators of both *Coreopsis* species.

Based on these results, it appears that *C. leavenworthii* and *C. tinctoria* are fully compatible and will produce seed naturally by insect pollination. Overall, it appeared that fitness of the interspecific hybrids was not affected in the vegetative traits, but reproductive fitness was affected in the F_1 and F_2 generations. Backcross populations

could additionally be assessed in the field to better understand the effects of interspecific hybridization on fitness. Because of the level of compatibility found, gene flow rates were lower than expected, possibly due to pollen competition or population model. These estimates do not take seed dispersal into account, and it is unknown how seed dispersal will affect gene flow. Regression analysis predicts that 0.10% gene flow may occur when the two species are 28 m away from each other, and 0.01% gene flow may occur when they are 60 m away. Finally, because of the negative effects found in the *C. leavenworthii-C. tinctoria* hybrids, pollen-mediated gene flow from *C. tinctoria* to *C. leavenworthii* should be prevented to protect the genetic diversity and integrity of *C. leavenworthii*.

APPENDIX: ENVIRONMENTAL CONDITIONS FOR GROWING YEARS DURING SEED PRODUCTION AND POPULATION EVALUATIONS

Table A-1. Environmental conditions during seed collection period for seed increase for the genetic diversity studies at the North Florida and Gulf Coast Research and Education Centers during 2007, 2008 and 2009.

Year	Location	Temperature (°C)	Relative Humidity (%)	Rainfall (in.)	Wind (mph)
2007	GCREC	23.2	71.08	0.09	8.04
	NFREC	23.1	70.15	0.07	5.40
2008	GCREC	23.3	70.75	0.11	7.00
	NFREC	20.9	71.19	0.09	5.32
2009	GCREC	21.6	72.72	0.10	7.63
	NFREC	21.6	76.88	0.25	5.42

Table A-2. Environmental conditions at monthly intervals during seed collection period for seed increase for the genetic diversity studies at the North Florida and Gulf Coast Research and Education Centers during 2007, 2008 and 2009.

Year	Location	Month	Temperature (°C)	Relative Humidity (%)	Rainfall (in.)	Wind (mph)
2007	GCREC	Mar	19.4	69	0.62	8.79
		Apr	20.7	68	3.65	7.98
		May	24.0	67	0.00	8.69
		Jun	25.7	78	5.07	6.87
	NFREC	Mar	16.3	68	1.71	4.98
		Apr	17.6	64	1.01	6.09
		May	23.1	59	0.99	6.06
		Jun	25.8	72	3.12	5.18
		Jul	26.5	77	4.63	4.79
		Aug	27.5	79	3.28	5.49
2008	GCREC	Mar	18.9	71	3.42	9.17
		Apr	20.6	65	0.03	7.46
		May	24.7	70	2.81	7.83
		Jun	26.1	77	5.85	5.52
	NFREC	Mar	14.4	68	3.66	6.53
		Apr	18.1	68	0.17	5.25
		May	23.0	70	1.48	5.80
		Jun	25.8	77	4.50	4.04
2009	GCREC	Mar	19.0	69	1.20	7.84
		Apr	21.5	70	0.00	8.66
		May	24.3	78	6.30	6.83
		Jun	26.9	79	4.80	6.07
	NFREC	Mar	15.3	73	7.10	5.99
		Apr	18.5	72	9.85	6.04
		May	22.9	81	5.11	5.63
		Jun	26.8	79	6.01	4.25

Table A-3. Temperature and relative humidity averages throughout the growing year during the evaluation of the seed increase populations at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010.

	AVG temp (°C)	MIN temp (°C)	MAX temp (°C)	Relative humidity (%)
2009	21.0	15.8	27.4	80.5
2010	24.3	18.1	31.2	76.2

Table A-4. Day length averages throughout the growing year during the evaluation of the seed increase populations at the Gulf Coast and Education Center, Wimauma, FL in 2009 and 2010.

	Overall (h)	Prior to 1st fl (h)	Flowering pd (h)	1 mo. prior to and including fl pd (h)
2009	11.9	13.4	11.44	11.86
2010	12.43	11.71	13.38	12.99

Table A-5. Soil and tissue nutrition components for *Coreopsis* plants grown in the field during the 2010 year at the Gulf Coast Research and Education Center, Wimauma, FL.

	P	K	Ca	Mg	Zn	Mn	Cu	pH	Fe	TKN
Soil sample	72.88	48.40	530.80	49.68	13.12	7.36	12.14	5.00	–	–
Plant tissue sample	3482	24360	16286	2929	127.70	103.60	21.40	–	105.80	45520

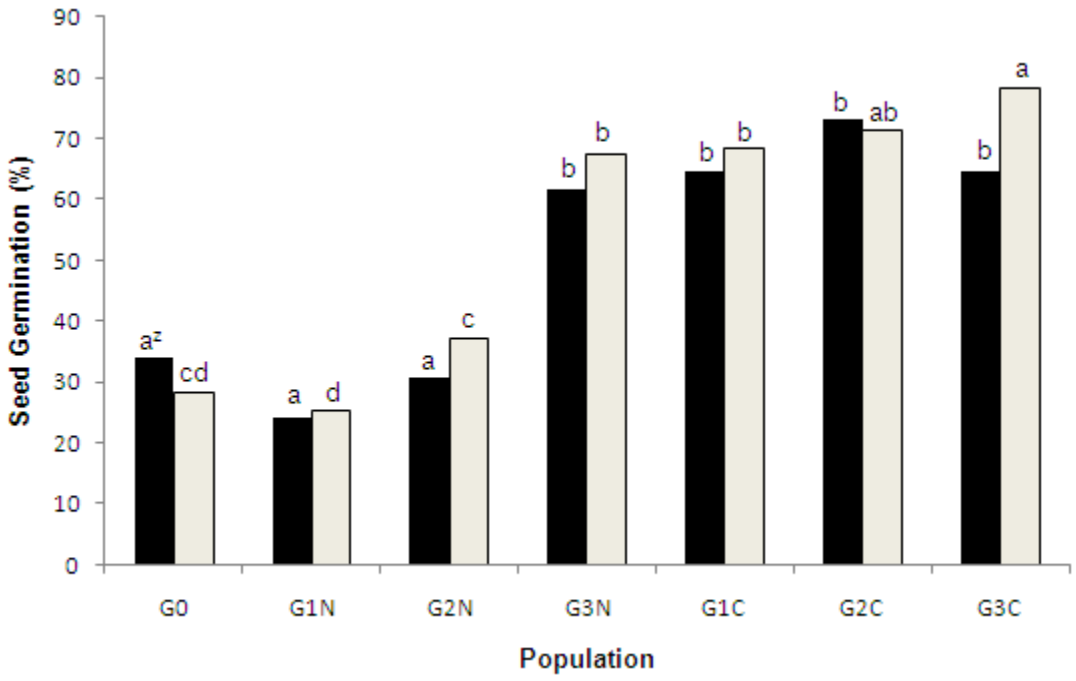

Figure A-1. Differences in seed germination of seven *C. leavenworthii* populations produced at two locations and sown at the Gulf Coast Research and Education Center, Wimauma, FL in 2009 and 2010. [z]Means within cultivar not followed by same letter are significantly different by Tukey's W Procedure at p≤0.05 using the percentages transformed by the arcsine square root method. Means for each year were statistically analyzed separately and cannot be compared to each other.

LIST OF REFERENCES

Abbott, R.J. 1992. Plant invasions, interspecific hybridization and the evolution of new taxa. TREE. 7(12):401-405.

Antonovics, J. 1976. The nature of limits to natural selection. Ann. Missouri Bot. Gard. 63:224-247.

Archibald, J.K., M.E. Mort, D.J. Crawford, and J.K. Kelly. 2005. Life history affects the evolution of reproductive isolation among species of *Coreopsis* (Asteraceae). Evolution. 59(11):2362-2369.

Arnold, M.A. and S.A. Hodges. 1995. Are natural hybrids fit or unfit relative to the parents? TREE. 10(2):67-71.

Arnold, M.L. 1996. Natural Hybridization and Evolution. Oxford University Press, New York.

Arnold, M.L., M.R. Bulger, J.M. Burke, A.L. Hempel, and J.H. Williams. 1999. Natural hybridization: How long can you go and still be important? Ecology. 80(2):371-381.

Arias, D.M. and L.H. Rieseberg. 1994. Gene flow between cultivated and wild sunflowers. Theor. Appl. Genet. 89:655-660.

Arriola, P.E. and N.C. Ellstrand. 1996. Crop-to-weed gene flow in the genus *Sorghum* (Poeaceae): Spontaneous interspecific hybridization between johnsongrass, *Sorhum halepense*, and crop sorghum, *S. bicolor*. Amer. J. Bot. 83(9):1153-1160.

Arriola, P.E. and N.C. Ellstrand. 1997. Fitness of interspecific hybrids in the genus *Sorghum*: Persistence of crop genes in wild populations. Ecol. Appl. 7(2):512-518.

Barton, N.H. 2001. The role of hybridization in evolution. Mol. Ecol. 10:551-568.

Bateman, A.J. 1947. Contamination of seed crops. J. Genet. 48:257-275.

Beckie, H.J. and L.M. Hall. 2008. Simple to complex: Modeling crop pollen-mediated gene flow. Plant Sci. 175:615-628.

Berg, E.E. and J.L. Hamrick. 1997. Quantification of genetic diversity at allozyme loci. Can. J. For. Res. 27:415-424.

Blears, M.J., S.A. de Grandis, H. Lee, and J.T. Trevors. 1998. Amplified fragment length polymorphism (AFLP): A review of the procedure and its applications. J. Ind. Microbiol. Biot. 21:99-114.

Booth, D.T. and T.A. Jones. 2001. Plants for ecological restoration: A foundation and a philosophy for the future. Native Plants J. 2(1):12-20.

Borner, A., S. Chebotar, and V. Korzun. 2000. Molecular characterization of the genetic integrity of wheat (*Triticum aestivum* L.) germplasm after long-term maintenance. Theor. Appl. Genet. 100:494-497.

Brewbaker, J.L. 1957. Pollen cytology and self-incompatibility systems in plants. J. Hered. 48:271-277.

Bryant, B. and B.L. Harper-Lore. 1997. Where wildflowers bloom, so does hope. US Dept of Transportation, Federal Highway Administration. Public Roads Online. Vol. 61(3).

Burton, P.J. and C.M. Burton. 2002. Promoting genetic diversity in the production of large quantities of native plant seed. Ecol. Restor. 20:117-123.

Cai, H.W. and H. Morishima. 2002. QTL clusters reflect character associations in wild and cultivated rice. Theor. Appl. Genet. 104:1217–1228.

Cain, M.L., B.G. Milligan, and A.E. Strand. 2000. Long-distance seed dispersal in plant populations. Amer. J. Bot. 87(9):1217-1227.

Chapman, M.A., D.G. Forbes, and R.J. Abbott. 2005. Pollen competition among two species of *Senecio* (Asteraceae) that form a hybrid zone on Mt. Etna, Sicily. Amer. J. Bot. 92(4):730-735.

Chebotar, S., M.S. Roder, V. Korzun, B. Saal, W.E. Weber and A. Borner. 2003. Molecular studies on genetic integrity of open-pollinating species rye (*Secalse cereal* L.) after long-term gene bank maintenance. Theor. Appl. Genet. 107:1469-1476.

Clausen J., D.D. Keck, and W.M. Hiesey. 1940. Experimental studies on the nature of species. I. Effect of varied environments on western North American plants. Washington (DC): Carnegie Institute. Publication 250.

Clewell, A.F. 1985. Guide to the vascular plants of the Florida panhandle. Univ. Press of Florida, Tallahassee, Fla.

Coyne, J.A. and H.A. Orr. 1998. The evolutionary genetics of speciation. Phil. Trans. R. Soc. Land. 353:287-305.

Crawford, D.J., E.B. Smith, and R.E. Pilatowski. 1984. Isozymes of *Coreopsis* section Calliopsis (Compositae): Genetic variation within and divergence among the species. Brittonia. 36(4):375-381.

Czarnecki, D.M., J.G. Norcini, and Z. Deng. 2007. Phenotypic diversity of *Coreopsis leavenworthii* Torr. and Gray (Asteraceae). Native Plants J. 8(1):45-57.

Czarnecki, D.M., M.N. Rao, J.G. Norcini, F.G. Gmitter, and Z. Deng. 2008. Genetic diversity and differentiation among natural, production, and introduced populations of narrowly endemic species *Coreopsis leavenworthii* (Asteraceae). J. Amer. Soc. Hort. Sci. 133:234-241.

Edmands, S. 2007. Between a rock and a hard place: Evaluating the relative risks of inbreeding and outbreeding for conservation and management. Mol. Ecol. 16:463-475.

Ellstrand, N.C. 1992a. Gene flow among seed populations. New Forest. 6:241-256.

Ellstrand, N.C. 1992b. Gene flow by pollen: Implications for plant conservation genetics. OIKOS. 63:77-86.

Ellstrand, N.C. 2003a. Current knowledge of gene flow in plants: Implications for transgene flow. Phil. Trans. R. Soc. Lond. 358:1163-1170.

Ellstrand, N.C. 2003b. Dangerous liaisons? When cultivated plants mate with their wild relatives. Johns Hopkins University Press, Baltimore.

Ellstrand, N.C., B. Devlin, and D.L. Marshall. 1989. Gene flow by pollen into small populations: Data from experimental and natural stands of wild radish. Proc. Natl. Aced. Sci. 86:9044-9047.

Ellstrand, N.C. and D.R. Elam. 1993. Population genetic consequences of small population size: Implications for plant conservation. Annu. Rev. of Ecol. Syst. 24:217-242.

Ellstrand, N.C., H.C. Prentice, and J.F. Hancock. 1999. Gene flow and introgression from domesticated plants into their wild relatives. Annu. Rev. Ecol. Syst. 30:539-563.

FDOT. 1995. Florida highway landscape guide. Florida Department of Transportation, Tallahassee, FL.

FDOT. 2006. 2006 FDOT design standards permanent erosion control. Florida Department of Transportation, Tallahassee, FL.

Ferdinandez, Y.S.N., B.E. Coulman, and Y.B. Fu. 2005. Detecting genetic changes over two generations of seed increase in an awned slender wheatgrass population using AFLP markers. Crop Sci. 45:1064-1068.

Foote, M. 1997. The evolution of morphological diversity. Ann. Rev. Ecol. Syst. 28:129-152.

Fu, Y.B., Y.S.B. Ferdinandez, A.T. Phan, B. Coulman, and K.W. Richards. 2004. AFLP variation in four blue grama seed sources. Crop Sci. 44:283-288.

Fulton, T.M., J. Chunwongse, and S.D. Tanksley. 1995. Microprep protocol for extraction of DNA from Tomato and other herbaceous plants. Plant Mol. Biol. Rep. 13(3):207-209.

Futuyma, D.J. 1979. Evolutionary Biology. Sinauer Associates, Inc., Sunderland, MA.

Gilman, E.F., J.G. Norcini and T. Howe. 2007. *Coreopsis leavenworthii*. FPS142.

Glenn, T.C. and N.A. Schable. 2005. Isolating microsatellites DNA loci. Method Enzymol. 395:202-222.

Goldstein, D.B. and D.D. Pollock. 1997. Launching microsatellites: A review of mutation processes and methods of phylogenetic inference. J. Hered. 88:335-342.

Grant, V. 1981. Plant Speciation. Columbia University Press, New York.

Greenleaf, S.S., N.M. Williams, R. Winfree, and C. Kremen. 2007. Bee foraging ranges and their relationship to body size. Oecologia. 153:589-596.

Hamrick, J.L. 1983. The distribution of genetic variation within and among natural plant populations, p. 335-348. In: S.M. Chambers and C.M. Schonewald (eds.). Genetics and wild population management. Addison-Wesley, New York.

Harper, J.L., J.N. Clatworthy, I.H. McNaughton, and G.R. Sagar. 1961. The evolution and ecology of closely related species living in the same area. Evolution. 15(2):209-227.

Harris, H. 1966. Enzyme polymorphism in man. Proc. of the Royal Soc. of London. 169:298–310.

Hartl, D.L. 2000. A primer of population genetics. 3rd ed. Sinauer Associates, Inc., Sunderland, MA.

Hausbeck, M.K., W.R. Quackenbush, and S.D. Linderman. 2002. Evaluation of cultivars of African daisy for resistance to powdery mildew. <http://plantpathology.msu.edu/labs/hausbeck/Publications/2003%20B&C%2018~O004.pdf>

Havens, K. 1998. The genetics of plant restoration: An overview and a surprise. Restor. Mgt. Notes. 16:68-72.

Heywood, V.H. 1993. Flower plants of the world. Oxford University Press, New York.

Hoekstra, F.A. and J. Bruinsma. 1975. Viability of Compositae pollen: Germination in vitro and influences of climatic conditions during dehiscence. Z. Pflanzenphysiol. Bd. 76:36-43.

Hufford, K.M. and S.J. Mazer. 2003. Plant Ecotypes: Genetic differentiation in the age of ecological restoration. TREE. 18(3):147-155.

Kabat, S.M., J.G. Norcini, and B. Deghan. 2007. Temperature and light affects germination ecology of commercially produced seeds of Leavenowrthi's coreopsis (*Coreopsis leavenworthii*). Native Plants J. 8(3):236-247.

Knapp, E. E. and K. J. Rice. 1994. Starting from seed: genetic issues in using native grasses for restoration. Restor. Mgt. Notes. 12:40–45.

Kumar, S., K. Tamara, and M. Nei. 2004. MEGA 3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings in Bioinformatics 5:150-163.

Langridge, P. and K. Chalmers. 2004. The principle: Identification and application of molecular markers, p. 3-54. In: H. Lorz and G. Wenzel (eds.). Mol. Marker Systems. Springer-Verlag, Berlin.

Levin, D.A. 1981. Dispersal versus gene flow in plants. Ann. Missouri Bot. Gard. 68(2):233-253.

Levin, D.A. and H.W. Kerster. 1969. The dependence of bee-mediated pollen and gene dispersal upon plant density. Evolution. 23(4):560-571.

Li, Y.C., A.B. Korol, T. Fahima, A. Beiles, and E. Nevo. 2002. Microsatellites: Genomic distribution, putative functions and mutational mechanisms: A review. Mol. Ecol. 11: 2453-2465.

Meyer, S.E. and S.B. Monsen. 1993. Genetic considerations in propagating native shrubs, forbs, and grasses from seed. p.47-54. In: T. D. Landis (technical coordinator). Proceedings, Western Forest Nursery Association Annual Meeting, Fallen Leaf Lake CA, September 14-18, 1992. USDA Forest Service General Technical Report RM-221.

Morgante, M. and A.M. Olivieri. 1993. PCR-amplified microsatellites as markers in plant genetics. Plant J. 3(1):175-182.

Mueller, U.G. and L.L. Wolfenbarger. 1999. AFLP genotyping and fingerprinting. TREE. 14(10):389-394.

Muller, A. and M. Kuhlmann. 2008. Pollen hosts of western palaearctic bees of the genus *Colletes* (Hymenoptera: Colletidae): The Asteraceae paradox. Biol. J. Linn. Soc. 95:719-733.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance form a small number of individuals. Genetics. 89:583-590.

Nei, M. 1987. Molecular Evolutionary Genetics. Columbia University Press, NY.

Norcini, J.G. 2002. Seed production of Leavenworth's *Coreopsis*. ENH868.

Norcini, J.G. and J.H. Aldrich. 2004. Establishment of native wildflower plantings by seed. Florida Cooperative Extension Service Publication. ENH 968.

Norcini, J.G. and A.H. Aldrich. 2007. Storage effects on dormancy and germination of native tickseed species. HortTechnology. 17(4):505-512.

Norcini, J.G. and J.H. Aldrich. 2008. Assessing viability of prevariety germplasm of native *Coreopsis* species. HortScience. 43(6):1870-1881.

Norcini, J.G., J.H. Aldrich, and F.G. Martin. 2006. Harvest year and fertilizer effects on seed production of Leavenworth's *Coreopsis*. J. Environ. Hort. 24(2):63-67.

Norcini, J.G., A.L. Frances, and C. Reinhart Adams. 2009. Establishment of Leavenworth's tickseed (*Coreopsis leavenworthii*) on roadside right-of-ways. ENH1104.

Norcini, J.G., M. Thetford, K.A. Klock-Moore, M.L. Bell, B.K. Harbaugh, and J.H. Aldrich. 2001. Black-eyed susan success: Your seed source is important. Greenhouse Prod. News. 11(2):58-60.

Osborne, J.L., A.P. Martin, N.L. Carreck, J.L. Swain, M.E. Knight, D. Goulson, R.J. Hale, and R.A. Sanderson. 2008. Bumblebee flight distances in relation to the forage landscape. J. Anim. Ecol. 77:406-415.

Parker, H.M. 1973. A biosystematic study of section Calliopsis of *Coreopsis* (Compositae). Univ. of Ark., Littlerock, PhD Diss.

Potts, B.M., W.C. Potts, and B. Cauvin. 1987. Inbreeding and interspecific hybridization in *Eucalyptus gunnii*. Silvae Genetica. 36:194-199.

Price, P.W. 1997. Insect Ecology 3rd Ed. John Wiley and Sons, Inc., New York.

Reed, D. H. and R. Frankham. 2003. Correlation between Fitness and Genetic Diversity. Conservation Biol. 17:230–237.

Rincker, C.M., R.G. May, H.H. Rampton, C.S. Garrison, and J.G. Dean. 1982. Genetic stability of three Italian ryegrass cultivars during seed multiplication in diverse environments. Crop Sci. 22:377-380.

Rogers, D.L. and A.M. Montalvo. 2004. Genetically appropriate choices for plant materials to maintain biological diversity. University of California. Report to the USDA Forest Service, Rocky Mountain Region, Lakewood, CO.

Rohlf, F.J. 2005. NTSYSpc numerical taxonomy and multivariate analysis system version 2.2. Exeter Software, Setauket New York.

Rukini, D. 2008. Dormancy in pre-variety germplasm of native *Coreopsis* species. University of Florida, Gainesville, PhD Diss.

Ryding, O. and K. Bremer. 1992. Phylogeny, distribution, and classification of the Coreopsideae (Asteraceae). Syst. Bot. 17:649–659.

SAS Institute, Inc. 1997. SAS Users Guide. SAS Inst., Inc. Raleigh, NC.

Schmitt, J. 1980. Pollinator foraging behavior and gene dispersal in *Senecio* (Compositae). Evolution. 34(5):934-943.

Sharma, J.N. 1971. The genetics, nature and occurrence of self-incompatibility and cross-incompatibility in four annual species of *Coreopsis* L. Montana State University, Bozeman, PhD Diss.

Sherff, E.E. 1955. *Coreopsis* and *Bidens*, p. 4-40. In: E.E Sherff and E.J. Alexander, Compositae-Heliantheae-Coreopsidinae. N. Amer. Fl. Series II. Part 2, New York Botanical Gardens, New York.

Simon, U. and A. Kastenbauer. 1979. Growth type and yield comparisons of forage species after seed multiplication in Germany and in the United States. II. Meadow Fescue, Timothy, and Perennial Ryegrass. Crop Sci. 19:209-213.

Sinclair, E.A. and R.J. Hobbs. 2009. Sample size effects on estimates of population genetic structure: Implications for ecological restoration. Restor. Ecol. 17(6):837-844.

Slatkin, M. 1987. Gene flow and the geographic structure of natural populations. Science. 236:787-792.

Smith, E.B. 1975. The chromosome numbers of North American *Coreopsis* with phyletic interpretations. Bot. Gaz. 136(1):78-86.

Smith, E.B. 1976. A biosystematic survey of *Coreopsis* in eastern United States and Canada. SIDA. 6(3):123-215.

Smith, E.B. 1983. Phyletic trends in sections Eublepharis and Calliopsis of the genus *Coreopsis* (Compositae). Amer. J. Bot. 70(4):549-554.

Smith, E.B. and H.M. Parker. 1971. A biosystematic study of *Coreopsis tinctoria* and *C. cardaminefolia* (Compositae). Brittonia. 23:161-170.

Song, Z.P., B.R. Lu, B. Wang, and J.K. Chen. 2004. Fitness estimation through performance comparison of F_1 hybrids with their parental species *Oryza rufipogon* and *O. sativa*. Ann. Bot-London. 93:311-316.

Stebbins, G.L. 1959. The role of hybridization in evolution. P. Am. Philos. Soc. 103(2):231-251.

Tadesse, M., D.J. Crawford, and E.B. Smith. 1995. Comparative capitular morphology and anatomy of *Coreopsis* L. and *Bidens* L. (Compositae), including a review of generic boundaries. Brittonia. 47(1):61-91.

Tesfaye, T., B. Getachew, and M. Worede. 1991. Morphological diversity in tetraploid wheat landrace populations from the central highlands of Ethiopia. Hereditas. 114:171-176.

Thiele, J., T. Hansen, H.R. Siegismund, and T.P. Hauser. 2010. Genetic variation of inbreeding depression among floral and fitness traits in *Silene nutans*. Heredity. 104:52-60.

Triplehorn, C.A. and N.F. Johnson. 2005. Borror and Delong's introduction to the study of insects. 7th Ed. Thomson Brooks/Cole, Belmont, CA.

Turesson, G. 1922. The genotypical response of the plant species to the habitat. Heriditas. 3:211-350.

USDA. 2011a. PLANTS profile *Coreopsis leavenworthii* Torr. and A. Gray Leavenworth's tickseed. United States Department of Transportation. 7 Feb. 2011.<http://www.plants.usda.gov/java/nameSearch?keywordquery=coreopsis+leavenworthii&mode=sciname>

USDA. 2011b. PLANTS profile *Coreopsis tinctoria* Nutt. Golden tickseed. United States Department of Transportation. 7 Feb. 2011. <http://www.plants.usda.gov/java/profile?symbol=COTI3>

van Treuren, R., R. Bulsma, N.J. Ouborg, and W. van Delden. 1993. The significance of genetic erosion in the process of extinction. IV. Inbreeding depression and heterosis effects caused by selfing and outcrossing in *Scabiosa columbaria*. Evolution. 47(6):1669-1680.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau. 1995. AFLP: A new technique for DNA fingerprinting. Nucl. Acids Res. 23(21):4407-4414.

Weising, K., H. Nybom, K. Wolff, and G. Kahl. 2005. DNA fingerprinting in Plants: Principles, methods, and applications. 2nd ed. CRC Press, Boca Raton, FL.

Whitten, J.L. 2002. Plant fact sheet Calliopsis *Coreopsis tinctoria* Nutt. USDA. <plants.usda.gov/factsheet/doc/fs_coti3.doc>

Wunderlin, R.P. 1998. Guide to the vascular plants of Florida. Univ. Press of Fla., Gainesville, Fla.

Wunderlin, R.P. and B.F. Hansen. 2004. Atlas of Florida vascular plants. Florida Center for Community Design and Research, and Institute for Systematic Botany, University of South Florida. 7 May 2011. <http://www.plantsatlas.usf.edu>

Yeh, F.C., R.-C. Yang, T.B.J. Boyle, Z.-H. Ye, and J.X. Mao. 1997. POPGENE, the user friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Canada.

Zane, L., L. Bargelloni, and T. Patarnello. 2002. Strategies for microsatellite isolation: A review. Mol. Ecol. 11:1-16.

BIOGRAPHICAL SKETCH

Sarah Smith was born in Leechburg, PA. She attended The Pennsylvania State University in State College, PA, where she graduated in May 2004 with a B.S. in horticulture. She then joined the Horticultural Sciences department at the University of Florida in August 2004 and received her Master of Science degree in December 2006. She then continued at the University of Florida to pursue her Doctor of Philosophy degree in the Horticultural Sciences Department, which was awarded in August 2011.