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Project R-002-09: Assessing Potential Loss of *Coreopsis leavenworthii* Genetic Diversity under Commercial Seed Production and Gene Flow from *Coreopsis tinctoria*

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Summary

The objectives of this project were to assess (1) the potential loss of genetic diversity (or genetic shift) during seed production of *C. leavenworthii*, and (2) potential gene flow from *C. tinctoria* to *C. leavenworthii*, two important issues in production and use of *C. leavenworthii* seeds.

G0 (generation 0, wild collection) seeds of *C. leavenworthii* were collected in summer and fall 2006. These seeds were increased to G1, G2 and G3 in two climatic zones (central and northern Florida) between 2007 and 2009, resulting in three increase populations from central Florida (G1C, G2C, and G3C) and three increase populations from northern Florida (G1N, G2N, and G3N). At the end of seed increase, seed emergence data were taken for G0 and all increase populations. Our results indicate that *C. leavenworthii* seeds can be stored for at least three years and seed emergence can be as high as 78.4%.

Two common garden studies were conducted, one in 2009 and one in 2010, to detect potential phenotypic changes that might have occurred during the three successive generations of seed increase. In each study, 525 individuals (75 per population and seven populations) were grown in the field, and data were taken from each individual on 12 characteristics, including plant height, plant dry weight, leaf type, days to flower, disk flower size, whole flower size, petal lobing, degree of petal overlap, number of ray petals per flower head, number of seeds per five seed heads, seed emergence, and powdery mildew severity. These common garden studies resulted in 121,600 data points (525 individuals × 12 characteristics × 2 years). Based on this data set, the increase populations had similar mean values with G0 over both years for nine out of the 12 characteristics evaluated. There were significant differences among populations for days to flower in 2010 but not in 2009, for degree of petal overlap over both years, and for seed production over both years. However, these differences were not consistent through generations or by site, thus the differences are likely to have been caused by random drift during seed increase. In principal component analysis, the populations were clustered primarily by year. 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 site. These results prompted us to conclude that three successive generations of seed increase in central or northern Florida did not cause significant population differentiation or genetic shift in

C. leavenworthii with respect to plant growth and development, leaf morphology, flowering and flower morphology, seed production and powdery mildew resistance.

Two approaches were taken to develop molecular markers: using genomic sequences from other Asteraceae and sequencing DNA of *C. leavenworthii* itself. The first approach resulted in a number of molecular markers that could reveal genetic diversity in both nuclear and chloroplast DNA. Application of these markers allowed the determination of genetic relationships between *Coreopsis* populations. Using the second approach, we obtained 384 *C. leavenworthii* DNA sequences and developed 45 SSR (simple sequence repeat) markers. The best 10 markers were used to assess the genetic diversity in the G0 population and the six increase populations. Genomic DNA was isolated from 385 individuals, 55 from each of the seven populations, and SSR marker data were obtained from 349 individuals.

Compared to G0, the increase populations each had 6.7% to 16.3% fewer marker alleles and 1.7% to 11.2% decrease in total genetic diversity (H_T). The decrease appeared to be stabilized at around 90% of G0's H_T. The change was similar between the two seed increase sites, that is, the decrease in H_T was independent of seed increase site. Genetic differentiation (G_{ST}) and genetic distance (between population pairs) increased slightly as the number of generations between each pair of populations increased. An UPGMA (unweighted pair group method with arithmetic mean) dendrogram constructed from the genetic distance matrix grouped G0, G1C, and G1N into one cluster and G2C, G2N, G3C, and G3N into another cluster, which suggested a subtle but consistent genetic differentiation as seed increase progressed successively. The clustering of seed increase populations was mainly by generation rather than by seed increase site. In the principal coordinate analysis (PCoA), individuals from G0 and the six increase populations were intermixed and did not form any definitive clustering by generation or site. This seems to indicate that although some genetic changes (or shifts) had occurred at the molecular level, the differentiation among the populations was relatively weak and the increase populations remained highly similar genetically to the original population.

In summary, molecular marker analysis results confirm that the genetic integrity of *C. leavenworthii* was maintained during the three generations of seed increase using the current production practices. It is likely that *C. leavenworthii* seeds can be increased for more generations without resulting in significant changes in genetic diversity or integrity. The linear relationship between population differentiation or genetic distance and seed increase generation

observed in this study suggests that later generations should be monitored to ensure that a satisfactory level of genetic diversity and integrity is preserved during seed production.

Controlled pollinations in the greenhouse showed that *C. tinctoria* and *C. leavenworthii* could hybridize and produce large numbers of viable hybrid seeds. When the two species were planted in the field, bees and wasps transferred pollen grains from *C. tinctoria* to *C. leavenworthii*, resulting in gene flow from the former to the latter, which, in turn, led to the production of interspecific hybrids under field conditions. The rate of gene flow from *C. tinctoria* to *C. leavenworthii* was affected by the distance between the two species. In the first experiment (2007-08), 5739 plants were examined. The highest gene flow rate observed was 3.9% when plants of the two species were 5 ft apart. Gene flow rate decreased to 0.2% as the separation distance increased from 5 to 200 ft. Gene flow (0.2%) occurred when the distance between the two species was up to 200 ft. Another field gene flow study was conducted in 2008 and 2009 to validate the above findings. Results from this study confirmed the strong effect of separation distance on gene flow as observed in the first test, but overall, the gene flow rates were slightly lower, and gene flow occurred within a shorter distance. The highest gene flow rate observed was 3.2% when the two species were planted 5 ft apart, and the lowest gene flow rate (0.3%) occurred when the two species were planted 50 ft apart. Gene flow was not observed when the separation distance was 100 ft or greater. To avoid potential depression from cross pollinations between the two species or gene pool contamination, gene flow should be taken into consideration when growers produce *C. leavenworthii* seeds or users plant the two species along roadsides or in landscapes.

Comparing completed research activities to the original proposal: We completed proposed research activities and conducted a substantial amount of extra work to better address the two major issues facing the Florida wildflower industry. Among the extra research activities were: 1) determining seed emergence of G0 and the six increase populations after storage; 2) assessing potential phenotypic changes of seven populations (525 plants per year, 2 years, and 12 characteristics); 3) increasing the number of pollen trap plots for gene flow study in each year by 50% (from 18 plots to 27 plots); 4) evaluating 11,350 plants in two gene flow studies (5,739 in the first and 5,611 in the second study), which was 5,950 more plants than proposed (100 plant per plot, 27 plots per year, and 2 years); and 5) identifying insect pollinators.

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The objectives of this project were to assess (1) the potential loss of genetic diversity (or genetic shift) during seed production of *C. leavenworthii*, and (2) potential gene flow from *C. tinctoria* to *C. leavenworthii*, two important issues in production and use of *C. leavenworthii* seeds.

1. Assessing the potential loss of genetic diversity from G0 to G3 during *C. leavenworthii* seed production in two climatic zones

1.1. Collection of G0 seeds (2006 and 2007)

Coreopsis leavenworthii generation 0 (G0) seeds were collected from the Reedy Creek Mitigation Bank, Polk County (USDA cold hardiness zone 9a, AHS heat zone 11) on July 1, 2006, and again on September 24, 2006. Seeds were dried, processed, and divided into four lots. One lot was stored at the United States Department of Agriculture Ornamental Plant Germplasm Center, Columbus, Ohio for long-term storage, two lots at the University of Florida/Institute of Food and Agricultural Science (UF/IFAS) North Florida Research and Education Center (NFREC) in Quincy, Gadsden County and one lot at the UF/IFAS Gulf Coast Research and Education Center (GCREC) in Wimauma, Hillsborough County. NFREC is in USDA cold hardiness zone 8b, AHS heat zone 9, and GCREC in USDA cold hardiness zone 9b, AHS heat zone 10. These seeds were used to produce G1 seeds.

1.2. Production of G1 seeds in two climatic zones (2007)

G1 seeds were produced both at GCREC and NFREC. At the GCREC, G0 seeds were sown on January 16, 2007, and seedlings were transferred to 4-cell packs on February 22, 2007, and potted up in #1containers on April 9, 2007. Potted plants (110) were spaced 12 inches apart, grown on benches, and irrigated through drip tubes (Figure 1). G1 seeds (G1C) were collected on June 16 and again on July 5, 2007.

At the NFREC, G0 seeds were sown on January 9, 2007, and seedlings were transferred to 4-cell packs on February 6, 2007. Seedlings were transplanted to a field seed production plot (12 inches on center) in a single 100-ft row on March 6, 2007. This seed production plot had

previously been covered with black woven landscape fabric; drip irrigation tubing was installed underneath the fabric. Plants were installed by cutting small holes in the fabric (Figure 2). The plot was surrounded by fencing to exclude armadillos and netting to exclude deer. G1 seeds (G1N) were collected in July and August.

1.3. Production of G2 seeds in two climatic zones (2008)

To produce G2 seeds at the GCREC, G1C seeds were sown on January 8, 2008, and seedlings were transferred to 4-cell packs on February 13, 2008, and potted up in #1containers on March 21, 2008. Potted plants (110) were grown outdoors in full sun and hand-watered daily (Figure 3). G2 seeds (G2C) were collected three times – May 28, June 9, and June 26, 2008.

To produce G2 seeds at the NFREC, G1N seeds were sown on January 1, 2008, and seedlings were transferred to 4-cell packs on February 1, 2008. Seedlings were transplanted to a field seed production plot (12 inches on center) in a single 100-ft row on March 6, 2008. Production plot characteristics were the same as in 2007. Harvest of G2 seeds (G2N) at the NFREC began on May 14, 2008 and continued to the end of June.

1.4. Production of G3 seeds in two climatic zones (2009)

To produce G3 seeds at the GCREC, G2C seeds were sown on January 7, 2009, and seedlings were transferred to 4-cell packs on January 27, 2009, and potted up in #1containers on February 23, 2009. Potted plants (more than 110) were grown outdoors in full sun and hand-watered daily (Figure 4). G3 seeds (G3C) were collected twice in May 2009 and once in June 2009.

To produce G3 seeds at the NFREC, G2N seeds were sown on January 7, 2009, and seedlings were transferred to 4-cell packs on February 6, 2009. Seedlings were transplanted to a field seed production plot (12 inches on center) in a single 100-ft row on March 9, 2009. Field plot conditions and cultural practices are the same as in 2007 (Figure 5). Seeds (G3N) were collected in May and June 2009.

Figure 1. *C. leavenworthii* in containers at the GCREC, Wimauma, Hillsborough County, for seed increase from G0 to G1C (2007).

Figure 2. *C. leavenworthii* planted at the NFREC, Quincy, Gadsden County, for seed increase from G0 to G1N (2007).

Figure 3. *C. leavenworthii* in containers at the GCREC, Wimauma, Hillsborough County, for seed increase from G1C to G2C (2008).

Figure 4. *C. leavenworthii* in containers at the GCREC, Wimauma, Hillsborough County, for seed increase from G2C to G3C (2009).

Figure 5. *C. leavenworthii* planted at the NFREC, Quincy, Gadsden County, for seed increase from G2N to G3N (2009).

Figure 6. *C. leavenworthii* plants (525) from G0 and six increase populations in the second common garden study to detect potential phenotypic changes that might have occurred during seed increase (2010)

1.5. Seed emergence of G0, G1, G2, and G3 populations (2009 & 2010)

Seeds from the seven populations were sown in 2009 to determine seed emergence. The emergence of G0 seeds was 34.0%. The emergence of G1C, G2C, and G3C seeds was between 64.6% and 73.2%, while the emergence of G1N, G2N, and G3N seeds was 24.0%, 30.8%, and 61.6%, respectively. To confirm the differences, seeds were germinated again in spring 2010. Similar differences have been observed. These data suggest that *C. leavenworthii* seeds can be stored for at least three years and seed emergence can reach as high as 78.4%.

1.6. Assessing potential phenotypic changes (2009 and 2010)

Seventy-five individuals per population and a total of 525 plants for the seven populations (G0, G1C, G1N, G2C, G2N, G3C, and G3N) were transplanted to field beds on September 3, 2009. Data were recorded between September 3, 2009 and January 15, 2010. The assessment was repeated in 2010. Seeds were sown on January 20, 2010, and seedlings were planted in ground beds on April 5, 2010. Data were recorded between April 19 and June 14, 2010 (Figure 6).

Data recorded were plant height (cm), plant dry weight (kg), leaf type, days to flower (DTF), disk flower size (DFS) (diameter, cm), whole flower size (WFS) (diameter, cm), petal

lobing, degree of petal overlap (DPO), number of ray flowers per flower head (NRP), number of seeds per five seed heads, emergence (%) of seeds collected from the field, and powdery mildew severity (PMS). Plant height was measured from the soil surface to the tallest point of the plant. Plant dry weights were recorded after whole plants were dried at 37.8°C and 20% relative humidity for at least 3 weeks. Leaf types were categorized on a 1 to 7 scale, where 1 was a simple leaf and 7 was the most complex leaf. In 2009, leaf type data were recorded 94 days after transplanting and in 2010 at 113 days. DFS and WFS were measured for five flowers of each plant. Petal lobing was rated on a 1 to 6 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). The DPO was rated on a 1 to 3 scale, where 1= petals oriented in a pinwheel fashion and not touching (G), 2= outside of the petals were touching but not overlapping (S), and 3= petals were overlapping (O). PMS was rated on a 1 to 10 scale, where 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. In 2009 PMS was recorded 113 days after transplanting and at 120 days in 2010. Seeds collected in the field in 2009 were sown on March 8-10, 2010 to determine seed emergence.

There were significant differences found between years but not among populations for plant height (Table 1). The mean plant height of G0 in 2009 was 72.3 cm (Table 1). The mean plant height of the six increase populations was 69.1 to 73.9 cm, or 95.6% to 102.2% of that of the G0. The mean plant height for G0 and the six increase populations in 2010 was 17.4% to 25.3% smaller than that in 2009. The mean plant height for the increase populations in 2010 was 97.2% to 107.6% of the mean height of G0, which was similar to the 2009 results.

There were not significant differences between years or among populations for plant dry weight (Table 1). The mean plant dry weight for G0 was 0.110 kg per individual (Table 1). The mean plant dry weight of the six increase populations was 0.099 to 0.116 kg, or 90.0% to 105.5% of that of G0.

Significant differences were found between years but not among populations for leaf type scores (Table 1). The mean leaf type score for G0 was 3.3 in 2009 and 3.3 to 3.4 for the six increase populations (Table 1). The mean leaf type score for G0 in 2010 was 4.2 in 2009. The mean leaf type score for the six seed increase populations was 3.8 to 4.2, similar to that of G0.

Significant differences were found for DTF between years and among populations in 2010 but not in 2009 (Table 1). In 2009 the mean DTF for G0 was 109.1 days (Table 1). The mean DTF for the six increase populations was 109.9 to 113.2 days, similar to that of G0. The mean DTF for G0 in 2010 was 7.1% less (101.3 days). The mean DTF for the six increase populations in 2010 was 98.9 to 103.5 days, similar to the mean DTF of G0. The significant difference detected among populations in 2010 was among G1C, G3C, G1N and G2N. The first three populations took fewer DTF (98.9 to 99.1 days), while the last population took significantly more DTF (103.5 days).

There were significant differences for DFS between years but not among populations (Table 1). The mean DFS for G0 was 0.79 cm in 2009 (Table 1). The mean DFS for the six increase populations ranged from 0.78 to 0.83 cm, similar to that of G0. The mean DFS for G0 grown in 2010 was 0.88 cm, 11.4% larger than the mean size in 2009. The mean DFS for the increase populations in 2010 was 97.7% to 102.3% of that of G0, similar to the 2009 results.

There were significant differences for WFS between years but not among populations (Table 1). The mean WFS was 3.3 cm for G0 in 2009 (Table 1). Similar mean WFS (3.4 to 3.5 cm) were recorded for the six increase populations. The mean WFS for G0 in 2010 was 9.1% less (3.0 cm) than in 2009. The mean WFS for the six increase populations in 2010 (2.9 to 3.0 cm) was similar to that of G0.

No significant differences were found between years or among populations for petal lobing (Table 1). The mean petal lobing score was 4.2 for G0 and 4.2 to 4.9 for the six increase populations (Table 1).

There were significant differences for the DPO between years and among populations (Table 1). The average rating for the DPO was 2.6 for G0 in 2009 (Table 1). G1C, G2C, and G3C had a mean rating of 2.7. The mean rating was 2.2 for G2N and 2.4 for G1N and G3N. G2N was significantly different from G1C, G2C, and G3C. The ratings decreased in 2010 compared to 2009 for all populations (Table 1). The mean rating for DPO in 2010 was 2.4 for G0, a 0.2 decrease from 2009. The mean rating for G1C, G2C, and G3C were from 2.3 to 2.4, a decrease of 0.3 to 0.4 from 2009. The mean ratings for G1N, G2N, and G3N were from 2.0 to 2.2, a decrease of 0.2 to 0.3 from 2009. G2N's mean rating was significantly different from G1C, G2C and G0's.

No significant differences were detected between years or among populations for the NRP per flower head (Table 1). The mean NRP per flower head was 8.1- for the G0 (Table 1). The mean NRP for the six increase populations was 8.0to 8.2.

There were significant differences between years and among populations for seed production. The mean number of seeds produced for G0 was 590.0 in 2009. Seed production for G1C, G2C, and G3C ranged from 540.9 to 652.4 seeds, not significantly different from that of G0 but G2C (540.9 seeds) was significantly different from G3C (652.4 seeds). G1N (425.8 seeds) produced significantly fewer seeds than G2N (570.5 seeds) and G3N (588.8 seeds) as well as G0 and the populations from central Florida. Seed production of G0 was the same in 2009 and 2010. Seed production of G1C (722.5 seeds), G2C (644.0 seeds), G3C (632.7 seeds), G1N (623.0 seeds) and G3N (691.3 seeds) was the same as G0, but G2N (757.9 seeds) was greater than G0.

There were not any significant differences found among populations for seed emergence in 2009 (Table 1). Mean seed emergence was 56.6% for G0 and 52.1% to 61.2% for the six increase populations (Table 1).

Significant differences were found between years but not among populations for PMS (Table 1). The mean severity score for PMS for G0 was 5.5 and 4.9 to 5.8 for the six seed increase populations in 2009 (Table 1). The mean PMS score was 3.0 for G0 and 2.5 to 3.2 for the six seed increase populations in 2010.

In summary, the increase populations had similar mean values with G0 over both years for nine out of the 12 characteristics evaluated. 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 site.

A dataset of 168 data points was assembled from the mean values of seven populations for 12 characteristics in two years (7 × 12 × 2). Principal component analysis of this dataset revealed that two principal components could account for 78.2% of the total observed variance. Based on these two principal components, the populations were clustered primarily by year (Figure 7). Within the 2009 cluster, the northern and the central Florida populations appeared to be separated. However, this clustering was not apparent 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 site

based on the phenotypic characteristics evaluated. Considering these results, we conclude that three generations of successive seed increase in central or northern Florida did not cause significant population differentiation or genetic shift in *C. leavenworthii* with respect to plant growth and development, leaf morphology, flowering and flower morphology, seed production, and powdery mildew resistance.

Table 1. Mean values of 11 characteristic for the seven *C. leavenworthii* seed increase populations s. Two common garden studies were conducted at GCREC in Wimauma, FL, one in 2009 and another one in 2010. Each population consisted of 75 individuals.

Plant Characteristic	Year	G0	G1C	G2C	G3C	G1N	G2N	G3N
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 rating	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 rating	Combined	4.2^{ns}	4.6	4.4	4.4	4.2	4.9	4.5
Petal overlap rating	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 emergence (%)	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

^{z}Significance tests were by Tukey's W Procedure at $p \leq 0.05$. ^{ns}Means in rows not significantly different by the tests.

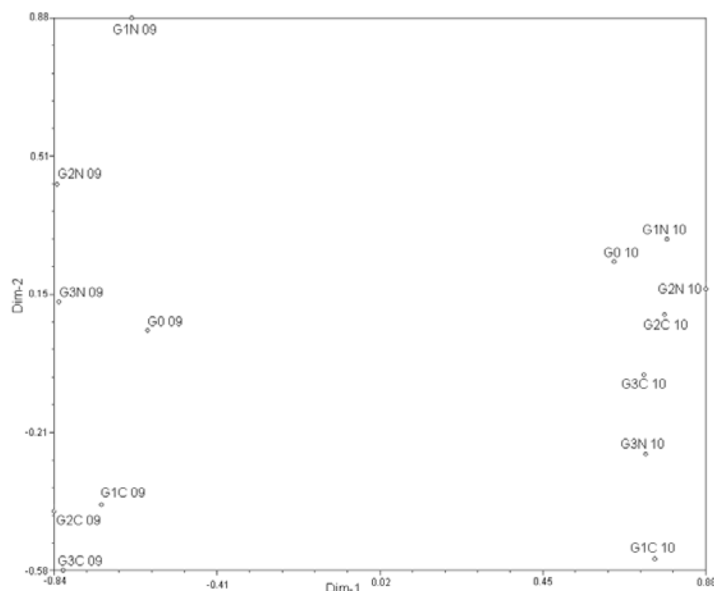

Figure 7. Principal component analysis of *C. leavenworthii* populations based on the population mean values of 12 characteristics. G0 and six increase populations (G1C, G2C, G3C, G1N, G2N, and G3N) were grown in 2009 (09) and again in 2010 (10) at the Gulf Coast Research and Education Center, Wimauma, FL.

2. Assessing potential changes of *C. leavenworthii* genetic diversity during seed production at the molecular level

2.1. Developing molecular markers (2007, 2008, 2009, and 2010)

Two approaches were taken to develop molecular markers that could provide high specificity, efficiency, and resolution (ability to differentiate homozygotes and heterozygotes, which are indistinguishable morphologically): using genomic sequences from other Asteraceae [UF/IFAS Citrus Research and Education Center (CREC) in Lake Alfred] and sequencing DNA of *C. leavenworthii* itself (GCREC in Wimauma). Using the first approach, we developed a number of molecular markers that could reveal genetic diversity in both nuclear and chloroplast DNA. Application of these markers allowed the determination of genetic relationships between *Coreopsis* populations (Figure 8). Using the second approach, we obtained 384 *C. leavenworthii* DNA sequences and developed 45 SSR (simple sequence repeat) markers. The best 10 markers

were used to assess the genetic diversity in the G0 population and the six increase populations. Genomic DNA was isolated from 385 individuals, 55 from each of the seven populations.

These markers amplified 104 alleles in the G0 individuals. Compared to the G0 population, the seed increase populations had 6.7% to 16.3% fewer alleles. The total genetic diversity (H_T) for the G0 population was 0.1736 (Table 2). The H_T for the G1C, G2C and G3C populations was 96.0%, 89.7%, and 91.8% of G0's H_T. The H_T for the G1N, G2N and G3N populations was 98.3%, 91.6%, and 88.8% of that of G0. Thus, these data indicate a 1.7% to 11.2% decrease in H_T within each increase population. The decrease appeared to be stabilized at around 90% of G0's H_T. The change was similar between the two seed increase sites, that is, the decrease in H_T was independent of seed increase site.

Genetic differentiation (G_{ST}) value between G0 and G1C, G2C, or G3C was 0.0244, 0.0394, and 0.0513, respectively, indicating a slight increase in population differentiation with each successive generation (Table 2). The G_{ST} value between G0 and G1N, G2N, or G3N 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. Genetic differentiation increased as the number of generations between each pair of populations increased (Figure 9).

The pairwise genetic distance (Nei, 1978) between G0 and G1C, G2C or G3C was 0.0080, 0.0141, and 0.0196, respectively (Table 2). The pairwise genetic distance between G0 and G1N, G2N or G3N 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 distance between each population pair increased as the number of generations between each pair increased (Figure 10).

An UPGMA (unweighted pair group method with arithmetic mean) dendrogram was constructed from the genetic distance matrix among these populations, and it clustered G1C and G1N with G0 and the two G2 populations with the two G3 populations (Figure 11). 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.

Table 2. Total genetic diversity (H_T), G_{ST}, and genetic distances for seven populations of *C. leavenworthii* produced in northern and central Florida.

	H_T	G0	G1C	G2C	G3C	G1N	G2N	G3N
G0	0.1736		0.0244^z	0.0394	0.0513	0.0238	0.0282	0.0399
G1C	0.1666	0.0080^z		0.0382	0.0367	0.0127	0.0231	0.0336
G2C	0.1558	0.0141	0.0133		0.0183	0.0354	0.0195	0.0130
G3C	0.1593	0.0196	0.0128	0.0050		0.0342	0.0346	0.0143
G1N	0.1706	0.0079	0.0030	0.0123	0.0119		0.0240	0.0292
G2N	0.1590	0.0092	0.0069	0.0052	0.0114	0.0075		0.0244
G3N	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.

Based on a total of 108 × 349 data points, a matrix of Apostol (simple match) distances among 349 individuals from the seven populations were calculated in the computer program NTSYSpc and used to conduct a principal coordinate analysis (PCoA). In the PCoA plot (Figure 12), individuals from G0 and the six increase populations were intermixed and did not form any definitive clustering by generation or site. This indicates that although some genetic changes (or shifts) had occurred at the molecular level, the differentiation among the populations was relatively weak and the increase populations remained highly similar genetically to the original population.

These results confirm that the genetic integrity of *C. leavenworthii* was maintained during the three generations of seed increase using the current production practices. It is likely that *C. leavenworthii* seeds can be increased for more generations without resulting in significant changes in genetic diversity or integrity. The linear relationship between population differentiation or genetic distance and seed increase generation observed in this study suggests that later generations should be assessed by common garden studies or molecular analysis to ensure that a satisfactory level of genetic diversity and integrity is preserved during seed production.

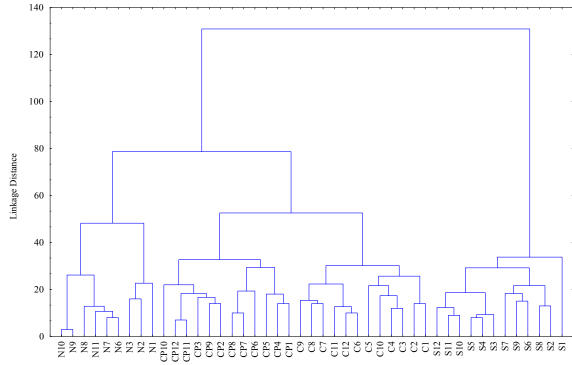

Figure 8. Molecular markers developed from other Asteraceae DNA sequences was used to assess genetic diversity and relationships in *Coreopsis* (2007 and 2008).

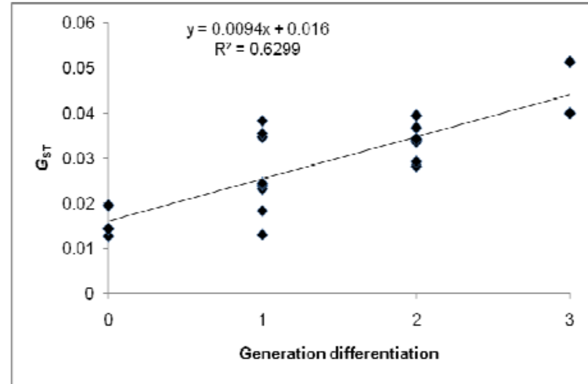

Figure 9. Relationship between population differentiation values (G_{ST}) and the number of generation between each combination of seven *C. leavenworthii* populations.

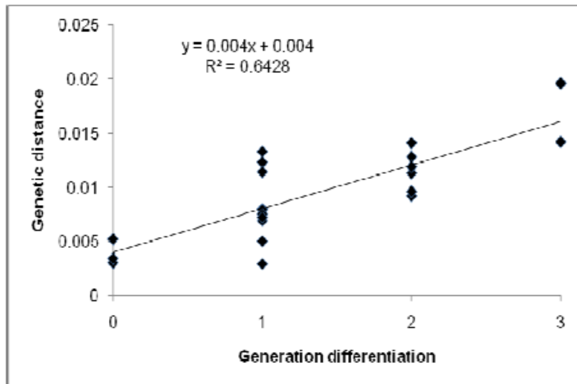

Figure 10. Relationship between genetic distance and the number of generation between each combination of seven *C. leavenworthii* populations.

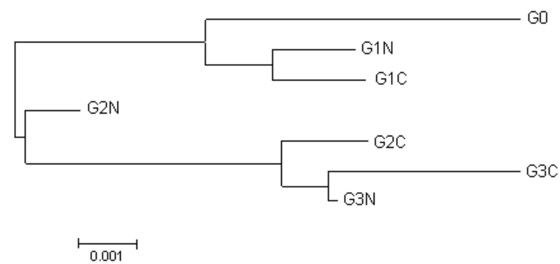

Figure 11. UPGMA dendrogram of G0 and six seed increase populations (G1C, G2C, G3C, G1N, G2N and G3N) of *C. leavenworthii* based on pairwise genetic distances.

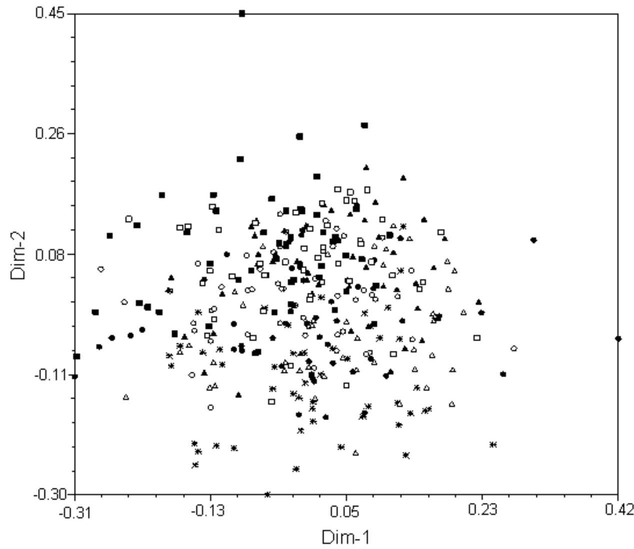

Figure 12. PCoA plot of 50 G0 (ж) individuals and 299 individuals of six seed increase populations (● G1C,▲ G2C,■ G3C,○ G1N,Δ G2N,□ G3N) of *C. leavenworthii*.

3. Assessing gene flow from *C. tinctoria* to *C. leavenworthii*

3.1. Evaluating the possibility of gene flow from *C. tinctoria* to *C. leavenworthii* (2006, 2007 and 2008)

Plants of *C. tinctoria* and *C. leavenworthii* were grown in the greenhouse at the GCREC. Pollen was collected from *C. tinctoria* and hand-pollinated onto ~30 flower heads of *C. leavenworthii* plants in July to September 2006. More than 3700 seeds were obtained, and on average, each flower head produced ~65 seeds. Approximately 600 seeds were sown on November 1, 2006, and 80% to 90% of these seeds germinated one month later (early December 2006). More than 97% of these seeds were shown to result from interspecific hybridization or gene flow from *C. tinctoria* to *C. leavenworthii*.

More than 200 additional flower heads of *C. tinctoria* and *C. leavenworthii* were cross-pollinated in June to August, 2007, and tens of thousands of seeds were produced. A total of 855 seeds from these pollinations were sown on October 31, 2007, and 82.5% (705) of them germinated by November 15, 2007 (two weeks after sowing). More than 99% of these seeds

were shown to result from interspecific hybridization or gene flow from *C. tinctoria* to *C. leavenworthii*.

These data indicate that the two species can hybridize and produce seeds of good viability (>82.5% emergence). According to the literature, these interspecific hybrids may show irregularities in pollen formation, have lower pollen stainability, and produce fewer seeds (Parker, 1973; Smith, 1976; Smith, 2011).

Parker, H.M. 1973. A biosystematic study of section Calliopsis of *Coreopsis* (Compositae). University of Arkansas, Littlerock, PhD Dissertation.

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

Smith, S.M. 2011. Assessing the potential effects of seed increase and interspecific hybridization on genetic diversity and fitness of *Coreopsis leavenworthii*. University of Florida, Gainesville, PhD Dissertation.

2.2. Identifying morphological markers to track gene flow events (2006, 2007 and 2008)

More than 300 *C. tinctoria* and *C. leavenworthii* plants were grown in the greenhouse at the GCREC and observed from December 2006 to April 2007 for differences in morphology. About 50 containerized plants of each species were grown at the NFREC and scrutinized for morphological differences as well. Two morphological markers were identified: the presence of 1-2 mm long trichomes ("hairs") on the petioles (leaf stems) (Figure 13), and the maroon spot on flower petals (Figure 14). The presence of trichomes can be evaluated as early as several weeks after seed germination, while it takes much longer (8 to 32 weeks) to evaluate the presence of spots on flowers. The presence of trichomes on *C. tinctoria* leaf petioles showed complex patterns of inheritance and it might be controlled by more than one gene locus. Because of this nature, trichomes are not a desirable morphological marker for tracking pollen-mediated gene flow from *C. tinctoria* to *C. leavenworthii*. The presence of the maroon spot is controlled by a single dominant nuclear gene in *C. tinctoria* and can be easily identified in the interspecific hybrids, thus it is a reliable morphological marker to identify pollen-mediated gene flow from *C. tinctoria* to *C. leavenworthii*.

In addition, we examined the presence or absence of wings on seeds (Figures 15 and 16) as an indicator of gene flow between the two species. The presence of wings seemed to have a complex inheritance pattern and not suitable for detecting pollen-mediated gene flow.

Figure 13. Trichomes on *C. tinctoria* leaf petioles.

Figure 14. Maroon spots on *C. tinctoria* flowers.

Figure 15. Wings on *C. leavenworthii* seeds.

Figure 16. Absence of wings on *C. tinctoria* seeds.

2.3. Testing the reliability of using maroon spots as indicators of gene flow from *C. tinctoria to C. leavenworthii* (2007 and 2008)

Testing the reliability of maroon spots and trichomes as gene flow indicators required controlled pollination of the two species (Figure 17), production of interspecific hybrids, and examination of the presence of *C. tinctoria*'s spots in the hybrids (Figure 18). Three experiments were conducted for this purpose.

Experiment 1: Seeds from two interspecific crosses between *C. leavenworthii* and *C. tinctoria* (cross # 605 and 606) (Table 3) were sown on November 1, 2006, and progeny were grown in containers in the greenhouse from November 2006 to April 2007. Altogether, more than 540 progeny were examined for the maroon spots on flowers. Greater than 97% of progeny expressed the spots (Table 3). This indicates that by examining the maroon spots at least 97% of the gene flow events from *C. tinctoria* to *C. leavenworthii* can be reliably identified.

Experiment 2: Another eight interspecific crosses (cross # 701 to 708) (Table 3) between *C. leavenworthii* and *C. tinctoria* were made in June to August, 2007. Seeds were sown on October 15, 2007; seedlings were grown in the greenhouse until January 30, 2008, and then planted in the field on January 30 and 31, 2008. Plants (a total of 688) were examined every two weeks for ~18 weeks by the end of May 2008. Of these plants, 242 flowered; 99 to 100% of these flowering interspecific hybrids showed the maroon spot on their flowers (Table 3).

Additional 24 interspecific crosses (cross # 709 to 732) between *C. leavenworthii* and *C. tinctoria* were made, and more than 500 progeny were examined in 2008-09. 100% of these progeny showed the maroon spot on their flowers.

In summary, data from these two experiments indicate that when gene flows from *C. tinctoria* to *C. leavenworthii* via pollen grains, greater than 97%, and up to 100%, of the gene flow events can be reliably detected using the maroon spot as a morphological marker, indicating an excellent utility and reliability of the morphological marker as an indicator of gene flow from *C. tinctoria* to *C. leavenworthii*.

Figure 17. Making controlled crosses to assess gene flow between *C. leavenworthii* and *C. tinctoria* (2007 and 2008).

Figure 18. Interspecific hybrids resulting from forced gene flow from *C. tinctoria* to *C. leavenworthii* (2007 and 2008).

Table 3. Testing the reliability of using maroon spots as a morphological indicator of gene flow from *C. tinctoria* to *C. leavenworthii*.

Cross #	Seed parent	Pollen parent	Total no. progeny examined	No. progeny		% interspecific hybrids showing maroon spots
				With maroon spots	Without maroon spots	
605, 606	COLE*	TINC**	546	530	16	97.1
701, 702, 703, 704	COLE	TINC	143	143	0	100.0
705, 706, 707, 708	TINC	COLE	100	99	1	99.0
Total			789	772	17	97.8

* COLE = *C. leavenworthii*; ** TINC = *C. tinctoria*

<u>2.4. Setting up field plots to detect gene flow events from *C. tinctoria* to *C. leavenworthii* (1st study, 2007)</u>

C. tinctoria and *C. leavenworthii* were grown in the greenhouse at the GCREC from January 16, 2007 to April 19, 2007. These plants were transplanted to the field (>700 feet long × 325 feet wide) in three separate blocks on April 20, 2007. In each block, the two species were separated in the field at nine distances: 5, 10, 25, 50, 100, 150, 200, 250, and 300 ft, with *C. tinctoria* plants as the pollen source and *C. leavenworthii* plants as the pollen traps. Blocks were separated by ~300 feet. Seeds were collected from the 27 trap plots (*C. leavenworthii*) three times, May 31, June 4, July 5, and July 10 (Figure 19). Seeds were dried, processed, and stored in July and August, 2007.

2.5. Determining the gene flow rates in the 1st study (2007 and 2008)

As insect pollinators visit the flower heads of the two *Coreopsis* species planted in the field in close proximity, these insects transfer some pollen grains (and genes within pollen) from *C. tinctoria* to *C. leavenworthii*, which would result in development of interspecific hybrid seeds. Therefore, to determine this type of gene flow requires germination of the seeds collected from the field gene flow study, growing-out of the seedlings to flowering, and close examination of flowers of each of the flowering plants.

Seeds collected from above (1st study) were sown in September 7, 2007. Seedlings were transferred to 72-cell trays on October 12, 2007. These plants were grown in a greenhouse in Gainesville (Figure 20). A total of 2,653 plants were examined from October 2007 to late May 2008. The data from these plants clearly indicated that pollen grains of *C. tinctoria* had been transferred by insect pollinators onto the *C. leavenworthii* flowers under natural field conditions. To better assess the effect of separation distance on the rate of natural gene flow from *C. tinctoria* to *C. leavenworthii*, we examined an additional 3,086 plants at the GCREC (Figure 21). Seeds from the 27 *C. leavenworthii* trap plots (1st study) were sown on January 10-11, 2008. Seedlings were transferred to 128-cell Speedling trays on February 15 and 18, 2008, and planted in the field on April 2, 2008. Figure 22 shows the gene flow rates as affected by separation distance. The highest gene flow (a rate of 3.9%) occurred when *C. leavenworthii* was planted 5 feet away (the shortest distance in this study) from the pollen source (*C. tinctoria*). As the separation distance increased from 5 ft to 100 ft, the gene flow rate decreased from 3.9% to 0.2%.

Figure 19. One of the three field blocks for field study of gene flow from *C. tinctoria* to *C. leavenworthii*. Seeds were being collected from one of the 27 pollen trap plots (2007).

Figure 20. Seedlings from the first gene flow study grown in the greenhouse to detect interspecific hybrids resulting from gene flow from *C. tinctoria* to *C. leavenworthii* (Gainesville, 2007)

Figure 21. Plants grown in the field to determine gene flow rates (2008).

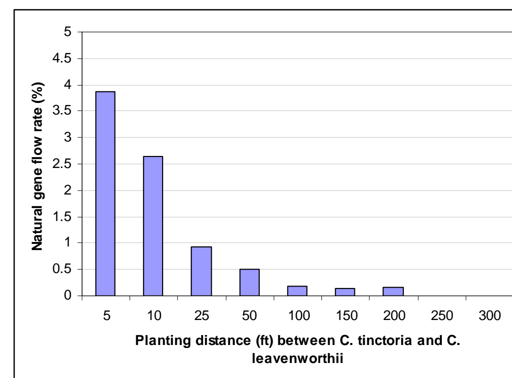

Figure 22. Gene flow rates as affected by separation distance between *C. tinctoria* and *C. leavenworthii* (2008).

2.6. Setting up the second field gene flow study to confirm the above findings (2008)

This field gene flow study (2nd study) (Figure 23) was intended to validate the above finding from the 1st test regarding the gene flow pattern and rate between the two species.

C. tinctoria and *C. leavenworthii* seeds were sown on January 9, 2008. Seedlings were transferred to 4-cell packs on February 13, 2008, and planted in the field on March 26, 2008.

The two species were again planted in the field at nine distances: 5, 10, 25, 50, 100, 150, 200, 250, and 300 ft, as they were in spring 2007. The plantings were replicated in three blocks, but with greater distances (500 to >1000 ft) between the field blocks to avoid potential interference among them. Each pollen source plot consisted of 48 plants, while each pollen trap plot consisted of 16 plants. A total of 96 *C. tinctoria* and 432 *C. leavenworthii* plants were installed in the field. Seeds were harvested from the 27 pollen trap plots on May 28, and again on June 25, 2008.

2.7. Determining the natural gene flow rates in the 2st study (2008, 2009)

The seeds collected from the second field gene flow study were sown on June 26 and July 7, 2008, respectively, into 6-inch azalea pots. Seedlings were transplanted into 128-cell Speedling trays on August 5-7, and then to field plots on September 12 and 15. More than 7,000 plants were transplanted and grown in the field (Figures 24 and 25). Data were recorded from October 20, 2008 to January 21, 2009. A total of 5,611 plants were examined. Out of these plants, 45 had the maroon spot, the morphological indicator of natural gene flow from *C. tinctoria* to *C. leavenworthii*. Results from this study confirmed the strong effect of separation distance on gene flow as observed in the first test, but overall, the gene flow rates were slightly lower, and gene flow occurred within a shorter distance, compared to the first gene flow study. The highest gene flow rate (3.2%) was observed when the two species were planted 5 ft apart, the lowest gene flow rate (0.3%) occurred when the two species were planted 50 ft apart, and no gene flow was observed when the separation distance was 100 ft or greater (Figure 26).

In summary, results from these studies showed that gene flow from *C. tinctoria* to *C. leavenworthii* occurred when the two species were separated as much as 200 ft. This factor needs to be considered when growing the two species in commercial seed production, highway beautification, landscape or ecological restoration in order to avoid gene flow between the two species and maintain the species integrity (or identity) of *C. leavenworthii*.

Figure 23. One of the three field blocks for the second field study of gene flow from *C. tinctoria* to *C. leavenworthii* (2008).

Figure 24. Seedlings from the second gene flow study were grown out in the greenhouse to detect interspecific hybrids resulting from gene flow from *C. tinctoria* to *C. leavenworthii* (2008).

Figure 25. Plants grown in the field to determine gene flow rates (second test, 2008-2009).

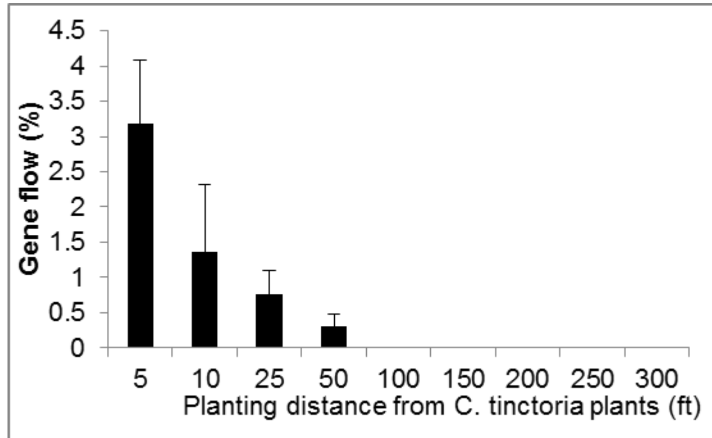

Figure 26. Rates of gene flow from *C. tinctoria* to *C. leavenworthii* detected in the second gene flow study (2008-2009).

2.8. Identifying insect species pollinating *Coreopsis* (summer and fall 2008)

Insects visiting *Coreopsis leavenworthii* or *C. tinctoria* flowers in the gene flow study field at GCREC were collected by hand on June 9, 2008. On September 25, 2008, insects visiting *Coreopsis* flowers in another field were collected. The collected insects were processed according to the Department of Plant Industry guidelines, and sent to the Florida Department of Agriculture and Consumer Services, Department of Plant Industry in Gainesville, FL for identification. The 14 insects collected in June belong to six families in the Order Hymenoptera (Table 4). The 15 insects collected in September belong to two genera in Hymenoptera, both of which were present in June 2008 (Table 4).

Table 4. Identified Insect Pollinators for Coreopsis

Family	Genus	Species	Common Name	Times collected in June 08	Times collected in Sept. 08
Colletiae	*Colletes*	sp.	Plasterer bee	1	
Eumenidae	*Euodynerus*	sp.	Potter wasp	1	
Halictidae	*Halictus*	*poeyi*	Halictid bee	4	14
Scoliidae	*Scolia*	*nobilitata*	scoliid wasp	3	
Sphecidae	*Philanthus*	*ventilabris*	Digger wasp	3	
Tiphiidae	*Myzinum*	sp.	Tiphiid wasp	1	1
Vespidae	*Polistes*	*bahamensis*	Paper wasp	1	

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