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Genetic variation in the chloroplast genome of a newly described Aster species, Chrysopsis delaneyi

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Genetic Variation in the Chloroplast Genome of a Newly Described Aster Species, *Chrysopsis delaneyi*

By

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ABSTRACT

The genus *Chrysopsis* (Asteraceae) contains eleven species native to Florida, including the newly described species, *Chrysopsis delaneyi*. Populations of this endemic plant species inhabit the Lake Wales Ridge (LWR) and the Atlantic Ridge (AR) of the Florida peninsula. Differences in morphology have been demonstrated within *C. delaneyi*, based on their locations. My objective was to determine the relationships between the LWR and the AR populations by analysis of chloroplast sequence and nuclear sequence variation. Approximately 160 samples of *C. delaneyi* and its sister species *C. scabrella* have been collected from fifteen sites throughout Florida. Six single base differences were detected, one insertion, and one variable short duplication. A total of four haplotypes (i.e.: groups that have different combinations of polymorphisms) have been found. For the most part, one haplotype is found in LWR populations and is indistinguishable from that found in *C. scabrella*. Another haplotype is found primarily in AR populations and is more similar to haplotypes

found in the more distantly related *C. highlandsensis* and *C. floridana*. One haplotype is found within populations of *C. scabrella*. The last haplotype in one AR population contains two polymorphic loci, one site is representative of the AR populations, and the other site is that of the LWR populations. Only one mixed population has been found, at the northern end of the AR range. These results are not consistent with taxonomic relationships inferred from morphological characteristics; hence the results suggest that chloroplast DNA (cpDNA) relationships may be the consequence of one or more instances of chloroplast capture.

INTRODUCTION

A key component in any conservation program is the preservation of genetic diversity of a species. This particular element of a conservation plan for endangered plant taxa can be one of the most difficult aspects to address, as human encroachment on sensitive areas often causes fragmentation of habitat, which can result in the isolation of populations. This fragmentation can lead to genetic drift in isolated populations, decreasing genetic variability of populations, and eventually decreased viability. Understanding the causes of genetic variation patterns in natural plant populations is an essential facet in conservation biology (Powell *et al.*, 1996B). Molecular markers are used to facilitate the determination of genetic variation because they are accurate and can quantify the degree of genetic diversity between, as well as within plant populations (Lakshmi *et al.*, 1997). The genetic information collected by such molecular techniques can then be used to develop and implement conservation recovery plans for endangered species.

The organelle genomes of mitochondria and chloroplasts have been used in animal and plant studies that include areas of evolutionary population biology such as migration patterns, historic events, and differentiation gradations of populations (Provan *et al.*, 2001). The unique characteristics of the chloroplast make it a useful tool for such studies. The circular structure of the chloroplast

DNA (cpDNA) in land plants is highly conserved, and the gene order is usually maintained. The cpDNA is divided into a large single copy region (LSC) and the small single copy region (SSC), with inverted repeats between the two regions. The LSC region is less conserved than the SSC region, making this region ideal for low taxonomic evaluations (Grivet *et al.*, 2001). The average size of the angiosperm chloroplast genome is approximately 148 kilobases (kb), providing a model size for restriction site analyses and direct sequencing comparisons (Olmstead *et al.*, 1994).

Several other features of the chloroplast genome are uniparental inheritance, and nonrecombination. Specifically, the chloroplast genome is inherited maternally in most angiosperms (Ferris *et al.*, 1997). Thus, directionality of seed and/or pollen dispersal can be followed, as well as their contributions to the overall genetic arrangement of plant populations (Provan *et al.*, 2001). Additionally, nonrecombination of the chloroplast genome demonstrates how the chloroplast is inherited as a unit, and is, for the most part, responsible for the lack of cpDNA variation in populations. Therefore, questions of gene introgression and sex-biased dispersal may be addressed by organellar polymorphism comparisons within and between populations (Wills *et al*., 2005).

The protein coding regions of chloroplasts are essential for photosynthetic activity as well as catabolic and metabolic functions. Thus, the frequency rate of mutations in the chloroplast genome is low, resulting in a lack of variation within these regions between species (Small *et al.*, 2005). However, noncoding regions of cpDNA, such as intergenic spacers and introns, are more likely to show a

greater amount of variation because they are less functional and more likely to mutate (Shaw *et al.*, 2005). Additionally, evolutionary changes of cpDNA such as small insertions and deletions of 1 – 100 base pairs (bp) have been documented.

From a conservation perspective, chloroplast markers have been the fundamental means used in previous phylogenetic studies, particularly those involving seed and pollen dispersal and their influence on the genetic structures of populations, establishment and factors of hybrid zones (McCauley, 1995), as well as tracing patterns of migrations (Huang *et al.*, 2002). Amplification of cpDNA by polymerase chain reaction (PCR) followed by restriction digests of the PCR product are useful tools for identifying intraspecific chloroplast polymorphisms (Provan *et al.*, 2001). These patterns of polymorphisms are more prevalent than previously thought, thus allowing the data to be used to evaluate the population level processes (McCauley, 1995).

Different types of molecular markers have been used to identify DNA polymorphisms. Restriction fragment length polymorphisms (RFLPs) are single or low copy probes that have been used to evaluate the amount of genetic variability in the chloroplast and nuclear genomes. Several drawbacks to this method include the use of large quantities of relatively pure DNA required for assay, as well as low levels of polymorphism detection in some plant species (Powell *et al.,* 1996B). Another technique uses arbitrary sequence markers known as randomly amplified polymorphic DNA markers (RAPD). This procedure involves amplification of genomic DNA at distinct loci by using random nucleotide sequence primers. The amplicons are then used to identify polymorphisms.

These fingerprints have been used to help determine the phylogenetic relationships within and between species (Rout, 2006). Restriction site mapping has been used widely in phylogenetic research as this straightforward method allows sampling of a number of sites for each enzyme for an indirect comparison of genetic variation. Additionally, variations of restriction sites located in noncoding regions render far more useful data pertaining to species phylogenies (Olmstead *et al.*, 1994).

Simple sequence repeats (SSRs), also known as microsatellites, are repetitive sequences of DNA, usually 1 – 6 bp repeated a number of times within the genome. In the chloroplast genome, the repeats are generally runs of T residues that vary in length, which result in the variations found within species (Powell *et al.*, 1996A). The motifs are generally more conserved in closely related taxa as compared to that of more distantly related taxa (Provan *et al.*, 2004). Several assumptions of SSR markers include selective neutrality, co-dominance, an equal distribution throughout the genome, and, these markers are effective in producing PCR products (Arnold *et al.*, 2002). In addition to detecting polymorphisms on loci, SSRs are used to test for new alleles. In comparison with RFLP methods, SSR only use small amounts of plant tissue and its use have uncovered more polymorphisms than previously thought existed in several plant species (Provan *et al.*, 2001).

Sequencing of the chloroplast genome is another valuable tool used in genetic variation studies because each nucleotide in the sequence can be compared. The amplified PCR products can be either sequenced directly, or

cloned first and then sequenced. Considerations for the use of sequencing for comparative studies should include sequence length of the fragment, a general understanding of the substitution rate within the sequence region (i.e.: is the sequence in a coding or noncoding region), and the ability of sequence alignment to other sequences (Olmstead *et al.*, 1994). This method can be used to resolve both higher-level and lower-level phylogenies, based on the regions examined.

Early phylogenetic studies on plant species based on cpDNA protein-coding regions had drawbacks. These regions are highly conserved, and have low mutation rates, limiting their use to high-level phylogenetic studies (Dumolin-Lapegue *et al.*, 1997). The non-coding regions, however, have demonstrated higher mutation rates, thus providing a more useful tool for taxonomic studies at lower levels (Shaw *et al.*, 2005). This finding, combined with the advances of more complete chloroplast genome sequencing and its conserved gene arrangement, allowed for the development of universal primers. These primers are targeted at the conserved flanking regions of the noncoding regions (Small *et al.*, 2005). Once amplified, the respective PCR products are generally small enough for direct sequencing or, if large enough, can be digested with restriction enzymes.

Another aspect of molecular marker use in conservation biology is defining units of flora and fauna for conservation purposes (Fraser & Bernatchez, 2001). In 1966, the U.S. Endangered Species Act (ESA) was initiated with the objective of protecting endangered fish or wildlife. However, the initial legislation was too restrictive, therefore requiring changes that were more suitable and more specific

for endangered species protection (Pennock & Dimmick, 1997). The concept of the evolutionarily significant unit (ESU) was introduced to acknowledge and implement a classification system for distinct groups lower than the already accepted taxonomic groups in order to preserve genetic diversity (Fraser & Bernatchez, 2001). Waples (1991) defined an ESU in order to distinguish the uniqueness of populations and, if found to be significant, warrant protection under the ESA. The definition states that an ESU is a population (or group of populations) that is isolated from and unable to reproduce with other populations of the same species, and plays an important role in the evolutionary heritage of the species. The catch with this definition is that even if a population can show divergence, either adaptive or genetic, it may not qualify as an ESU if it does not demonstrate phylogenetic uniqueness, and will not be protected under the ESA (Young, 2001).

Other definitions of ESUs include those of Ryder (1986); Dizon *et al*., (1992); Avise (1994); Moritz (1994); Vogler & DeSalle (1994); and Crandall *et al.,* (2000) (Fraser & Bernatchez, 2001). Ryder's 1986 definition, which initiated the ESU, was set in place to characterize subdivisions of a broad group of a particular species, whose genetic features are unique and noteworthy for the preservation of current, as well as future populations of species. This definition too, had a catch, because it did not include rules for implementation (Fraser & Bernatchez, 2001). Dizon *et al.* (1992) looked at allele frequencies for their definition, specifically, concentrating on the divergence of these frequencies between populations of species. Other factors causing reproductive isolation

were considered which included localization of populations, species behavior, morphology and selection. Avise's (1994) definition states that ESUs should be grouped by similar gene phylogenies which constantly result in population distinction into subgroups based on genetic characterization and geographical levels.

Moritz (1994) states that the primary rationale for defining ESUs is to acknowledge and maintain the evolutionary lineage of a unit in order for genetic diversity to be passed along. Moritz based his definition of an ESU on demonstrating that there actually is the existence of a particular type of genetic difference, rather than just looking at the quantity. Genetic differences are illustrated by the distribution of nuclear, mitochondrial or chloroplast alleles within populations (DeWeerdt, 2002). Therefore, Moritz points out several characteristics that should be included when describing an ESU:

- Members of ESUs should not share a common ancestor with any other individuals of another population, a term known as reciprocal monophyly.
- Nuclear allelic frequencies should demonstrate great divergence.
- The time period for reciprocal monophyly to occur in populations that have been separated should be 4N generations.
- As a result of high substitution rates compared to nuclear genomes, organellar genomes are anticipated to reach this state at a significantly fast rate.

- Nuclear allele frequencies must be examined in concert with organellar frequencies and demonstrate a good proportion of divergence in order to determine correctly phylogenies based on both data sets.

Vogler & DeSalle (1994) take an alternative approach to ESUs. They define a conservation unit by character features that cluster groups together, a theory known as Phylogenetic Species Concept (PSC). This concept looks at the ancestral condition for discrete clusters in order to define it as a conservation unit. Additionally, PSC can be examined by population aggregation analysis (PAA) to recognize the orders of related species, but specifically to include groups that are joined by fixed character states (Fraser & Bernatchez, 2001). Crandall *et al.* (2000) view defining distinct populations as a complete process involving different degrees of gene flow resulting in a group's individual uniqueness from adaptation through events such as genetic drift and natural selection. This principle is based on a null hypothesis of a population's uniqueness, then, if applicable, the population is categorized for protection (Fraser & Bernatchez, 2001).

Florida Plants and Habitats

There are 4,189 plant taxa currently listed in the state of Florida, of which 230 taxa are endemic. This diversity of plant species is attributed to Florida's geographic location, as well as its size and shape. Starting from the Atlantic and

Gulf Coastal Plains, Florida stretches down into the Caribbean, and is surrounded on the east coast by the Atlantic Ocean and the Gulf of Mexico on the west coast. This allows temperate plant species to thrive in north and central Florida and sub-tropical and tropical plant species to grow in the southern part of Florida. Additionally, the mild climate of Florida allows non-native (exotic) species to adapt and become naturalized, which makes up 31% of the recognized taxa of its flora (Wunderlin & Hansen, 2000).

Florida scrub habitats are composed of sandy soils that are nutrient-poor. These conditions are ideal for woody, xeric vegetation, and are pre-disposed to infrequent high-intensity fires, which limit the plant diversity of this environment (Myers & Ewel, 1990). There are numerous plant species that are prevalent to the scrub habitat and are not found in any other habitat. Fifty-five species are presently listed at the federal level as endangered or threatened, and 22% are on the State of Florida's list. Rare scrub species are limited to the Lake Wales Ridge of Florida, possibly as result of the ancient landscape and previous island-type environment along the ridge tops (Myers & Ewel, 1990).

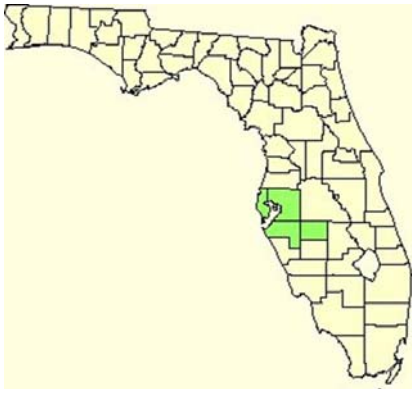
There are several characteristic layers in scrub habitats. The shrub layer consists of six commonly occurring species, listed in order of their presence and abundance: myrtle oak or scrub oak (*Quercus myrtifolia*, *Q. inopina*), saw palmetto (*Serenoa repens*), sand live oak (*Q. geminata*), Chapman's oak (*Q. chapmanii*), rusty lyonia (*Lyonia ferruginea*), and Florida rosemary (*Ceratiola ericoides*). The ground layer includes gopher apple (*Licania michauxii*), beak rush (*Rhynchospora megalocarpa*), milk peas (*Galactia* spp.), *Andropogon*

floridanum, and *Panicum patentifolium*, the lichens British soldier moss (*Cladonia leporina*), *C. prostrata*, *Cladina evansii*, and *C. subtenuis* (Myers & Ewel, 1990). *Chrysopsis floridana,* the Florida golden aster, is restricted to a limited number of scrub habitats in Hillsborough, Manatee, Pinellas, and Hardee Counties. Other species of *Chrysopsis* include *C. scabrella*, which is found throughout the state, and *C. highlandsensis*, which is found primarily in the central interior sections of the state in Glades, Highlands, and Polk Counties (Figure 1).

Chrysopsis delaneyi

Chrysopsis delaneyi is a short-lived perennial herb found throughout several counties in the Lake Wales Ridge (LWR), and the southeast Atlantic Ridge (AR). Populations on the LWR occur in southern Lake, western Osceola, eastern Polk, and northwestern Highlands Counties, specifically in turkey oak sandhills and longleaf pine environments. Extant populations are small and fragmented. The AR populations inhabit sand pine and hickory scrub environments along southern Brevard, Indian River, St. Lucie, Martin, Palm Beach, and Broward Counties. There are numerous populations found along US highway 1 from Jonathan Dickinson State Park north to Hobe Sound, mainly growing on open dunes. A few populations are found on the Orange County Uplands, some located at close to the University of Central Florida (DeLaney *et al.*, 2003) (Figure 1).

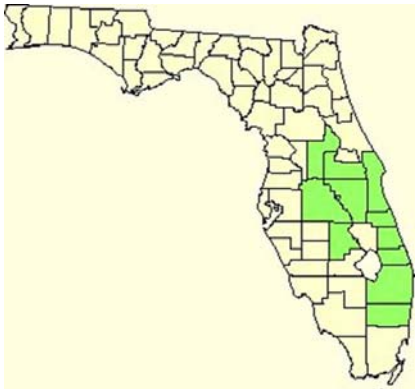
Figure 1. Distribution of Four *Chrysopsis* Species In Peninsular Florida



C. floridana

C. scabrella

C. highlandsensis



C. delaneyi

In addition to the variable habitats *C. delaneyi* occupies, populations also display differences in morphology (Figure 2). Initially, *C. delaneyi* was identified as *C. scabrella* but has since been found to differ from that species. Both species share similar morphology, such as yellow-green colored leaves, small capitula, short, sparse trichomes, thin linear leaves, and overall small plant size compared to the silver-green colored sericeous-tomentose leaves, large capitula, thick

linear leaves, and larger plant size of *C. highlandsensis*. However, some AR plants are somewhat larger than the LWR plants, and have thicker trichomes, thicker, more densely sericeous-tomentose linear leaves, and larger capitula, and are more robust than LWR populations. Based on this observation, the possibility exists that this species may typify several races that have genetically adapted to certain environmental conditions. As a result of the natural landscape of the upland ridge system, certain populations of *C. delaneyi* have become isolated, thus allowing them to their uniqueness due to allotropy (Delaneyi *et al*, 2003). Additionally, some populations may have adapted to environmental changes within their own habitats resulting in variation in the genetic structure within the species.

Chrysopsis delaneyi has a woolly-pubescent basal rosette, with rosette leaves (7.0)10.0-16.0(18.0) cm long and (0.8)1.5-2.7(3.5) cm wide. These leaves are broadly spatulate, oblanceolate, or narrowly lanceolate to nearly linear. Rosettes with stems can be up to 15 cm tall. These mostly grow for two or more years before flowering, and often branch into clusters of multiple rosettes after the first year of growth. The stems are (0.8)1.0-1.2(1.8) cm in diameter near the base, (0.6)0.8-1.2(1.5) m tall, densely leafy, stipitate-glandular or glandular hirsute. The flower head is corymbiform or paniculiform, compact to moderately open, moderately branched, measuring 30-70(200), (2.4)4.2(4.6) cm in diameter (including ray straps). The branches are stout and densely viscid stipitate-glandular. *Chrysopsis delaneyi* flowers from mid-November to early January,

except for Orange County populations, which begin flowering in October (Delaney *et al*, 2003).

Overview

The objective of this study is to investigate the chloroplast and nuclear genome distributions within populations of *C. delaneyi* to determine the amount of differentiation within local species. The morphological differences of this plant species based on population locations raise some questions about the evolutionary processes taking place. Speculation that *C. delaneyi* is a species composed of several ecotypes has been suggested by DeLaney *et al*. (2003). Therefore, investigating the possibility that *C. delaneyi* is an evolutionarily significant unit is included in this study.

Based on the chloroplast data, I have found that the cpDNA variation distribution of *C. delaneyi* is consistent with the existence of two ESUs. Most of the LWR populations display differentiation from the AR populations, but similar to *C. scabrella*. Additionally, there is a mixed population group containing both LWR and AR haplotypes and a population that contains two polymorphic loci consistent with one of each of the AR and LWR populations. The relationships with other *Chrysopsis* species are not consistent with morphological data. Implications with respect to species conservation and management will be discussed.

Figure 2: Examples of *C. delaneyi* Populations and Habitats
A: UCF Campus in Orange County
B: Jonathon Dickenson State Park

2-A

2-B

MATERIALS AND METHODS

Population Sites

In November, 2003, one hundred sixty three leaf samples of both *C. delaneyi* and *C. scabrella* were collected from fifteen sites (Table 1). Leaf samples were collected by removing two leaves from each flowering plant and placing them into plastic bags containing silica gel desiccant. The bags were subsequently labeled with an abbreviated code and number corresponding to the collection site (Table 2). The bags were then stored at -20^{o} C until the DNA could be extracted.

Table 1: Number of Sites and Plants Sampled

Taxon	Number of Sites	Plants Collected
C. scabrella	4	34
C. delaneyi (LWR)	6	67
C. delaneyi (AR)	5	62

Table 2: Geographic Coordinates and Abbreviated Codes of Collection Sites

Population Abbreviation	Site Designation	County	Latitude	Longitude
APA	Avon Park	Highlands	27.61508N	81.51349W
APP	Avon Park	Highlands	27.60191N	81.50578W
1-708	NW US1 – 708	Martin	27.06102N	80.1377W
BSNPRK	Babson Park	Polk	27.83356N	81.52695W
ESUSI	East Side US1 – hundreds	Martin	27.00865N	80.10195W
FL708	SE Bridge Rd	Martin	27.0581N	80.14137W
HGH	Highlands Avenue	Highlands	27.57053N	81.49587W
HTCWAT	NE Inters. Hatchineha & Watkins	Polk	28.03448N	81.52817W
IRCL	Daytona Blvd. W of 3rd St	Brevard	27.86725N	80.49953W
RSLND	Roseland	Indian River	27.83025N	80.4791W
ScTIT1	S. Side FL50	Brevard	28.55427N	80.8201W
ScYH2	W. Side 441 N Yeehaw	Osceola	27.71323N	80.91133W
STLUS1	W. Side US1	St. Lucie	27.49442N	80.3447W
UCF	UCF W. Entrance Univ Blvd	Orange	28.59788N	81.20537W
EEE	Triple E	Lake	28.61647 N	81.71317 W
EMRLD	W side Emerald Drive	Hernando	28.50953 N	82.1814 W

DNA Extractions

DNA extractions were performed using a Plant DNA Isolation Kit by Roche Diagnostics Corp. following the manufacturer's protocol, with exceptions to the amount of buffers added as follows: Buffer 1 from 150 µl to 300 µl; Buffer 2 from 10 µl to 20 µl; and Buffer 3 from 50 µl to 100 µl.

PCR Amplifications

All cpDNA used for PCR amplifications were diluted to a 1:10 concentration. All PCR amplifications were carried out as a 50 µl volume reaction containing 5 µl of 10X magnesium-free reaction butter (50mM potassium chloride, 10mM Tris-HCl, 0.1% Trition X-100) supplied by Promega, 5 µg (0.25 µl) bovine serum albumin (BSA), 0.25mM each dNTP, 50 pmol primer and 10-50 ng template DNA (1 µl), 1U Taq DNA polymerase (Promega), and 3 µl of 1.5mM of 1X magnesium chloride. The PCR thermocycling conditions and primer sequences for the various PCR reactions are found in Table 3. All agarose gels were run in 1x TBE Buffer (Tris, boric acid, EDTA, pH 8) and visualized with ethidium bromide staining.

The initial chloroplast markers used in this study were universal primers specific for the *trn*L CD region. These markers were used because previous analysis had revealed the presence of two restriction site polymorphisms, detected by digestion with Alu1 and DpnII (Walker and Cochrane, unpublished). A 2% agarose gel was used for electrophoresis.

Consensus chloroplast microsatellite primers (ccmp) specific for the intron of the *trn*G gene (ccmp3) were tested and subsequently used in this study. These primers not only target SSRs, but also have been used effectively in cpDNA variation studies, particularly in angiosperms (Weising *et al.*, 1999). The number of poly (A) microsatellites found in the amplicon cause the variations in species. These residues, which are less common in the organellar genome

compared to nuclear genome, are generally 20 bp long (Weising *et al*, 1999). A 3-1/2% agarose gel was used for electrophoresis.

A noncoding region of the *trn*K gene in the chloroplast genome was examined using matK6f and matK5r primers. Phylogenetic studies for both interspecific and intraspecific have relied on noncoding cpDNA regions focused in the LSC. The conserved nature of the genes flanking these regions, especially in angiosperms, allows for easy and effective primer design for lower-level taxonomic studies (Shaw *et al.*, 2005). A 2% agarose gel was used for electrophoresis.

Table 3: Thermocycling Conditions for PCR Reactions

Primer	Sequence	Size (bp)	PCR Conditions
trnC trnD	5’ – CCA GTT CAA ATC TGG GTG TC – 3’ 5’ – GGG ATT GTA GTT CAA TTG GT – 3	~500	5 minute at 94º C, 30 cycles of 30 seconds at 94º C, 30 seconds at 50º C, 1 minute at72º C, 5 minutes at 72º C
ccmp3f ccmp3r	5’ – CAG ACC AAA AGC TGA CAT AG – 3’ 5’ – GTT TCA TTC GGC TCC TTT AT – 3’	~120	5 minute at 94º C, 30 cycles of 1 minute at 94º C, 1 minute at 52º C, 1 minute at 72º C, 5 minutes at 72º C
matK6f matK5r	5’ – TGG GTT GCT AAC TCA ATG G – 3’ 5’ – GCA TAA ATA TAY TCC YGA AAR ATA AGT GG – 3’	~1500	5 minutes at 95º C, 35 cycles of 1 minute at 95º C, 1 minute at 50º C (ramp of 0.3º C/second), 5 minutes at 65º C, 5 minutes at 65º C

To examine the nuclear genome, the intron region of the Actin 1 gene was used (Slomba *et al.*, 2004). PCR reactions were performed using Actin 1 forward primers (5' – CCC GAA TTC CTT GTT TGC GAC AAT GGA AC – 3') and Actin 1 reverse primers (5' – CCC GAA TTC ACA ATT CCA TGC TCA AT – 3') to produce a 316 bp fragment. The thermocycling protocol was 1 minute at 95° C, 35 cycles of 15 seconds at 95° C, 30 seconds at 48° C, and 90 seconds at 72° C, followed by 10 minutes at 72° C. PCR amplified products were run on a 2% agarose gel in 1X TBE and visualized with ethidium bromide staining.

Restriction Enzyme Digests

The amplified products of the *trn*L CD region, as described above, were subsequently digested with both Alu1 and DpnII enzymes in separate reactions. The first reaction combined 1 µl of Alu 1, 1 µl of Alu 1 buffer (10mM Tric-HCl, 50mM NaCl, 10mM $MgCl_2$, 1mM Dithiothreitol, pH 7.9 at 25° C), and 8 µl (25-38ng/µl) of PCR product at 37° C for 2 hours. The second reaction combined 1 µl of DpnII, 1 µl of DpnII buffer (100mM NaCl, 50mM Bis Tris-HCl, 10mM , $MgCl_2$, 1mM Dithiothreitol, pH 6.0 at 25° C), and 8 µl (25-38ng/µl) of PCR product at 37° C for 2 hours. The digested PCR products were then run on a 2-1/2% agarose gel for the Alu1 digests and a 3% agarose gel for the DpnII digests and visualized with ethidium bromide staining.

Gel Extractions

DNA samples from the matK and Actin 1 PCR amplifications were extracted from 1% agarose gels and purified for subsequent direct sequencing and cloning reactions, respectively, using a QIAquick Gel Extraction Kit (Qiagen Corp.) following manufacturer's protocol. Approximately 45 µl of each PCR product was loaded into the gel. For the Actin 1 samples, 1 gel volume of isopropanol was added to the sample tubes and mixed to increase the yield of DNA fragments. The DNA was then stored at -20^{o} C.

Sequencing Reactions

The thermocycler protocol for direct sequencing of the matK amplifications was 30 cycles of 20 seconds at 96^{o} C, 20 seconds at 50^{o} C, and 4 minutes at 60^{o}C. Reactions were carried out in 10 µl volumes containing 25-38 ng of DNA, 0.0 µl or 1.5 µl sterile water, 1.6 pmol of matK5r primer, and 4 µl QuickStart Master Mix (supplied with kit). The sequencing reactions were followed by ethanol precipitation of products first by adding 4 µl of stop solution (50% volume of 3M sodium acetate and 50% of 100 mM EDTA) and 1 µl of glycogen solution (supplied with kit) to each reaction tube. This was followed by the addition of 60 µl of -20^{o} C 95% ethanol/water (v/v) to each tube, which was then mixed by pipetting. The tubes were then centrifuged at 14,000 rpm for 15 minutes. The

ethanol was removed from the tubes by pipetting. This was followed by 200 µl of -20° C 70% ethanol/water (v/v) added to each tube and centrifuged at 14,000 rpm for 5 minutes. The ethanol was removed from the tubes by pipetting and the 70% ethanol/water (v/v) step was repeated one more time. The pellets were dried at room temperature for 20 minutes, then resuspended in 40 µl of Sample Loading Solution (SLS) (supplied with kit).

PCR Fragment Cloning

Several single band products from ccmp3 and Actin 1 PCR products were cloned using the TA Cloning® Kit for Sequencing (Invitrogen™ Corp.) following manufacturer's protocol. 50 µl from each transformation reaction were spread on pre-warmed LB medium plates, each containing 30 µg/ml of kanamycin. The plates were incubated at 37° C overnight. Following manufacturer's protocol, one colony of each was picked and cultured overnight in LB broth containing 30 µg/ml of kanamycin. Plasmid minipreps were performed with Purelink Quick Plasmid MiniPrep kit (Invitrogen™ Corp.) following manufacturer's protocol. The plasmid DNA was first heated for 3 minutes at 96° C and then cooled to room temperature before adding the rest of the reagents. Sequencing reactions were carried out in 10 µl volumes containing 17ng/ul of plasmid DNA, 1.6pmol of M13 primer supplied with kit (M13F: 5' – CTG GCC GTC GTT TTA C – 3'; M13R: 5' –

CAG GAA ACA GCT ATG AC – 3'), and 4 ul QuickStart Master Mix (supplied with kit). This was followed by ethanol precipitation as described above.

Nucleotide Sequence Comparisons

Sequences derived from PCR amplifications with ccmp3 primers were used to search for other plant species that contained similar sequences (Table 4). A Basic Local Alignment Search Tool (BLAST) found at the National Center for Biotechnology Information (NCBI) website was used. An alignment was created with these sequences, along with several *C. delaneyi*, *C. scabrella*, and *C. highlandsensis* sequences using ClustalW in the Molecular Evolutionary Genetics Analysis (MEGA) software program (Kumer, Tamura, Nei, 1993-2005).

Table 4: BLAST Sequences of ccmp3 Region

Accession #	**Family**	**Genus**	**Species**
AY871258.1	Rosaceae	*Prunus*	*ilicifolia*
AY727221.1	Asteraceae	*Trilisa*	*paniculata*
AY727220.1	Asteraceae	*Carphephorus*	*corymbosus*
AY727509.1	Solanaceae	*Solanum*	*physalifolium*
AY727222.1	Asteraceae	*Eupatorium*	*rotundifolium*
AY727513.1	Caryophyllaceae	*Minuartia*	*uniflora*
DQ352338.1	Altingiaceae	*Altingia*	*obovata*

RESULTS

Restriction Enzyme Digests

A total of 122 *C. delaneyi* and 30 *C. scabrella* samples were digested with both Alu 1 and Dpn II. The results show that the AR populations have restriction sites for both of these enzymes. Alu 1 digestion produced two bands in the AR samples, compared to one band in the LWR and *C. scabrella* samples when visualized on a 2-1/2% agarose gel. DpnII digestion produced a smaller band in the AR samples, compared to the slightly larger bands in the LWR and *C. scabrella* samples (Figure 3 and Figure 4).

Sequencing Reactions

The PCR products from the ccmp3 amplifications were examined by electrophoresis, using a 3-1/2% agarose gel. The LWR and *C. scabrella* samples showed larger bands by approximately 20 bps when compared to the AR samples (Figure 5). Subsequently, 5 samples were first cloned and then sequenced. Two samples were from the IRCL mixed population, one sample was a *C. scabrella* and two were AR samples. The results showed one insertion and one variable short duplication in the *C. scabrella* sample, as well as in one of the

IRCL samples (Table 5). The duplication sequence contains six A residues compared to the seven just upstream of it.

Figure 3: Electrophoresis of Alu 1 Digest. Lane 3, AR sample with double bands. The other lanes are all LWR samples with a single band.

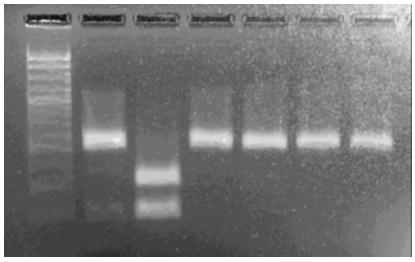


Figure 4: Electrophoresis of DpnII Digest. Lane 2 is AR sample with smaller band. Lanes 3 is LWR sample with a slightly larger band

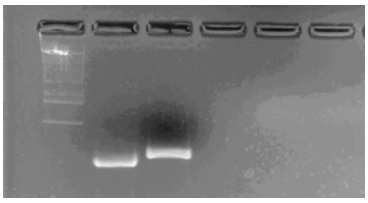


Figure 5: Electrophoresis of ccmp3 PCR.
Lanes 2 and 3 are AR samples with small bands.
Lanes 4 – 8 are LWR and *C. scabrella* samples with larger bands

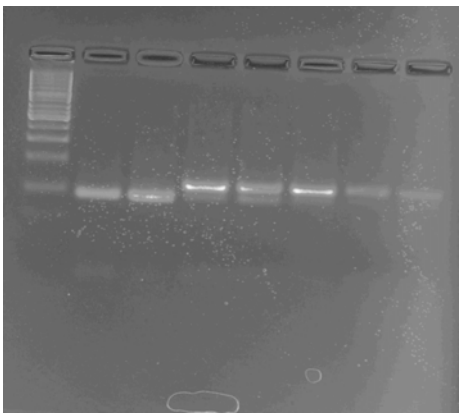


Table 5: ccmp3 Insertion Sequences

```
                                      105                                                 145
                                       ↓                                                   ↓
AR #APA3F    TATGGAAAAT GGATATAT-T GCTA------ ---TGTGAAC CAACTTACAA AAAAATGATA CCCA------ ----------
AR #FL708-3F .......... .........-. ....------ ----------. .......... .......... ....------ ----------
AR #IRCL49F  .......... .........G. ....------ ----------. .......... .......... ....------ ----------
AR #IRCL50F  .......... .........-. ....GATTTA AGA.......  .......... .......... ....ACTTAC AAAAAATGAT
SC #SCCL35F  .......... .........-. ....GATTTA AGA.......  .......... .......... ....ACTTAC AAAAAATGAT

AR #APA3F    -----TAACA
AR #FL708-3F -----.....
AR #IRCL49F  -----.....
AR #IRCL50F  ACCCA.....
SC #SCCL35F  ACCCA.....
```

PCR fragments amplified with the matK primers were sequenced to examine cpDNA variations. In total, 134 samples comprised of 109 *C. delaneyi*, 21 *C. scabrella*, and 4 *C. highlandsensis* were directly sequenced. Variations between LWR and AR populations, within *C. scabrella* populations, and within one AR population were discovered in four positions (Table 6). The first variation appears in the *C. scabrella* scYH2 populations, where there is a single base change of G compared to an A in the other populations of *C. scabrella* as well as in the AR, LWR, and *C. highlandsensis* populations. The second variation distinguishes not only the LWR and AR populations, but also the *C. scabrella* and the *C. highlandsensis* populations. Both the LWR and *C. scabrella* populations have a T base at this locus, while AR and *C. highlandsensis* have a C base. Additionally, this similarity occurs again with the fourth locus. The LWR and *C. scabrella* have a C base, and the AR and *C. highlandsensis* have a T base. The third variation tends toward the same pattern as the fourth, however, in the AR RSLND populations, rather than retaining the G base as with the other AR populations, there is a base change to a C. The IRCL populations demonstrate a mixture of both the AR and LWR haplotypes.

Table 6: cpDNA Variations Found with matK Primers at Four Base Positions (BP)

Population	BP 212	BP 270	BP 329	BP 332
LWR	G	T	C	C
AR	G	C	G	T
C. highlandsensis	G	C	G	T
C. scabrella	G	T	C	C
C. scabrella (YH2)	A	T	C	C
AR (RSLND)	G	C	C	T
IRCL (5 samples)	G	T	C	C
IRCL (5 samples)	G	C	G	T

Two samples that were cloned from the actin 1 PCR products, one from an AR population and one from a LWR population, were sequenced. A BLAST search of each of these sequences resulted in actin gene coding sequences or partial coding sequences in a number of plant families. An alignment between the two sequences shows two regions that are suspect of an insertion/deletion event (Table 7).

BLAST Results

The results of the ccmp3 BLAST found various plant families that had some similarities in their sequences with the *C. delaneyi*, and *C. scabrella* sequences (Table 8). The alignment of these sequences aligned 4 bps of the outgroups with the 9 bps of the first insertion site of the LWR and *C. scabrella* samples, with the exception of the Rosaceae family. The 7 outgroups do not show a duplication of the bases at the second site as seen in the LWR and *C. scabrella* samples, however they do contain 18 to 21 similar base pairs between them.

Table 7: Actin 1 Sequence Alignment with AR and LWR Populations

```
Identical=. Missing=? Indel=-;

                                                                    50
                                                                    ↓

#AR_STLU68F CCCGAATTCC TTGTTTGCGA CAATGGAACT GGAATGGTTA AGGTACCGAA TAATAGAATC TCTGCACACA CATTGATCTA
#LWR_UCF7F  .......... .......... .......... .......... ......TC.- ---------T .TGCA...TG ...CAG--..



#AR_STLU68F AAAGTTACGC CCCAAAGTTA AATGCTTGTC TATATATAAA TGATGTTAAT TTGCAGGCTG GATTTGCGGG TGATGATGCA
#LWR_UCF7F  G..A..CAAA --TG.TT.C. G...G.A... ..A.C-C..G .A.CT..TTG .......... .T.....T.. A........T



#AR_STLU68F CCACGAGCTG TGTTCCCAAG TATTGTGGGT CGTCCACGCC ATACTGGTGT GATGGTTGGC ATGGGCCAAA AAGATGCATA
#LWR_UCF7F  ...A.G.... .......... C.....A..C ..A..T..T. .C.....A.. .........A .......... .......T..



#AR_STLU68F TGTTGGTGAT GAGGCTCAGT CCAAGAGAGG TATCTTGACA CTGAAGTACC CGATTGAGCA TGGAATTGTG AATTCGGGAA
#LWR_UCF7F  ......A..C .......... .......G.. ......A..T .....A.... .A........ .......... ..........

                       333
                        ↓
#AR_STLU68F GGGCGAATTC GT-----TTA AACCTGCA-- -GGACTAG-- ----
#LWR_UCF7F  .......... .CGGCCGC.. ..TTCA..TT C.CC...TAG TGAG
```

Table 8: ccmp3 Sequence Alignment with Outgroups

```
Identical=. Missing=? Indel=-;


#AR_APA3F                 ATGGAAAATG GATATAT-TG CTA------- --TGTGAACC AACTTA-CAA AAAAATGATA CCCA------ ----------
#AR_FL708-3F              .......... .......-.. .......... --........ .......... .....-.... .....------ ----------
#Mixed_IRCL49F(AR)        .......... .......G.. .......... --........ .......... .....-.... .....------ ----------
#Mixed_IRCL50F(LWR)       .......... .......-.. ...GATTTAA GA........ .......... .....-.... ....ACTTAC AAAAAATGAT
#C_scabrella_SCCL35F      .......... .......-.. ...GATTTAA GA........ .......... .....-.... ....ACTTAC AAAAAATGAT
#Rosaceae_Prunus          GGAA..G.AT TT.G.T.CCA .CGAGCTAAA ACAA.TTGT. G.TG.CT.T. GT...CC.A. GT..TTGTTT AATAGCTATT
#Asteraceae_Trilisa       ..A.TT..G. .G.C.T.CG. T.TGATT--- --CA.ATT.. G.T----A.. ...CT.T..T T.AT---TTA AAGGATTGAA
#Asteraceae_Carphephorus  ..A.TT..G. .G.C.T.CG. T.TGATT--- --CA.ATT.. G.T----A.. ...CT.T..T T.AT---TTA AAGGATTGAA
#Solanaceae_Solanum       ..A.TT..A. .G.CCT.CG. T.TGATT--- --C..ATT.. G.T----... ...CT.T..T T.-T---TAA AAGGATTAAA
#Asteraceae_Eupatorium    ..A.TT..G. .G.C.T.CG. T.TGATT--- --CA.ATT.. G.T----A.. ...CT.T..T T.AT---TTA AAGGATTGAA
#Caryophyllaceae_Minuartia..A.TT..A. .G.GCCAAG. T.TGATT--- --AC.ATT.. ...-----..T T..CT.T..T T.T.---AAT AAGGAATTAA
#Altingiaceae_Altingia    -.A.TT..G. .G.CCT.CG. T.TGATT--- --CA.ATT.. G.T----... ...CT.T..T T.-T---TAA AAGGATTTAA


#AR_APA3F                 -----TAA--
#AR_FL708-3F              -----...--
#Mixed_IRCL49F(AR)        -----...--
#Mixed_IRCL50F(LWR)       ACCCA...--
#C_scabrella_SCCL35F      ACCCA...--
#Rosaceae_Prunus          TTGCT.C.AT
#Asteraceae_Trilisa       TCCTT.-.--
#Asteraceae_Carphephorus  TCCTT.-.--
#Solanaceae_Solanum       TCCTT.-T--
#Asteraceae_Eupatorium    TCCTT.T.--
#Caryophyllaceae_MinuartiaTCCCT.----
#Altingiaceae_Altingia    TCCTT.-.--
```

DISCUSSION

The objective of this study was to examine selected chloroplast and nuclear genes in order to determine variations within populations of *C. delaneyi* because of the morphological differences demonstrated among these populations. The primary focus was to resolve the question that *C. delaneyi* may be composed of several ecotypes. The results of the chloroplast data show strong evidence to support this. Additionally, direct sequencing of the cpDNA showed within species variation of *C. scabrella*. A total of four haplotypes have been discovered with this study (Table 9).

The presence or absence of the restriction sites in *C. delaneyi* populations clearly delineate the 2 groups. The results of the ccmp3 sequencing substantiate the newfound relationship between AR and *C. highlandsensis*, and between LWR and *C. scabrella.* The most likely evolutionary event occurring is that of duplication of an upstream sequence and insertion in the LWR and *C. scabrella* species. Intraspecific variation detected by the matK sequencing isolated the scYH2 population, located at Yeehaw Junction, from the rest of the *C. scabrella* populations. This single base difference was the only intraspecies specific variation found in all of the groups tested. Although preliminary, the alignment of the actin intron sequences from a LWR and an AR sample does suggest that

there may be sufficient variation within this region to be informative with respect to phylogenetic relationships in the genus.

The predicted relationship between populations of the AR and the LWR *C. delaneyi* has not been found in this study. Instead, the cpDNA distribution points to a relationship between LWR and *C. scabrella,* and then reveals an unexpected relationship between AR *C. delaneyi* and *C. highlandsensis* (Figure 6). A key to understanding how these different relationships may have evolved is to look at the possible interactions between these species as well as spatial patterns of the various populations of *Chrysopsis*.

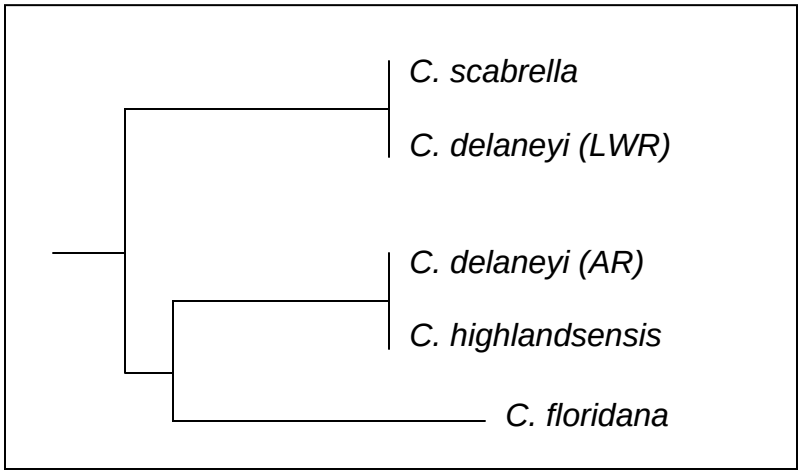
Table 9: Table of Haplotypes

Haplotype	**Variable Site**				**Found in Populations**
	trnL	**trnL-trnF***	**ccmp3****	**trnK-matK**	
1	GGA	-	+	GTCC	BSNPRK, EEE, EMRL, HTCWAT, IRCL, OK, scCL, scTI1, UCF
2	ACG	+	-	GCGT	AP, ESUS1, FL708, IRCL, STLUS1
3	GGA	-	+	ATCC	scYH2
4	ACG	+	-	GCCT	RSLND

*trnL-trnF:
+ Presence of Restriction Site
- Absence of Restriction Site

**ccmp3:
+ Presence of Insertion/Duplication Sequence
- Absence of Insertion/Duplication Sequence

Figure 6: Phylogenetic Tree of *Chrysopsis* Based on Chloroplast Data



The theory of introgression within the *C. delaneyi* species has been suggested by Semple (personal communication). Possible introgression patterns include the chloroplasts of *C. scabrella* incorporating into LWR populations or *C. highlandsensis* chloroplasts integrating into AR populations, and through backcrossing, each population group maintaining their chloroplast genotype respectively. Cases of chloroplast capture have been documented in several plant families such as Saxifragaceae (*Mitella)* and Asteraceae (*Helianthus*, and *Artemisia*). An early study conducted by Rieseberg *et al.* (1990) examined the relationship between *Helianthus annuus* ssp. *texanus* and *Helianthus debilis* ssp. *cucumerifolius* using a combination of chloroplast and nuclear ribosomal DNA markers. They concluded that the most likely scenario was chloroplast capture of *H. debilis* ssp. *cucumerfifolius* by *H. annuus* ssp. *texanus*. Another study by Kornkven *et al.* (1999) looked at cpDNA restriction site variations to determine phylogenetic relationships between 11 species of a woody shrub, *Artemisia* sect.

Tridentatae. They found that 2 unrelated species, *A. californica* and *A. filifolia* were grouped in the *Tridentata* clade as a result of chloroplast capture. Finally, Okuyama *et al*. (2005) examined three regions of DNA by direct sequencing in order to explain discrepancies found in the nuclear and chloroplast phylogenies of *Mitella*. The chloroplast data were derived from the noncoding region of the *trnL-F* gene and the *matK* gene, as well as the external transcribed spacer (ETS) and internal transcribed spacer (ITS) regions of the nuclear ribosome. Grading the patterns of introgression from these regions found that the chloroplast region was the most widespread, followed by the ITS region. The ETS region did not demonstrated any pattern of introgression. The conclusion for the differences in the ITS and ETS patterns of introgression was nonuniform concerted evolution.

Successful chloroplast capture is dependent on a number of different factors. The initial obstacle would be the adaptability of the donated chloroplast to the host species. A model presented by Tsitrone *et al.* (2003) suggests that the chloroplast genes and the nuclear genes would be incompatible with each other, giving rise to cytoplasmic male sterility (CMS), either partial or complete in the first generation. This response to introgression would, in turn, increase the fitness of the female by allocating the energy from pollen production to seed production. Thus, breeding systems such as random mating and partial-selfing must be taken into consideration with this model, along with several assumptions, which include a single diploid nuclear locus and a single cytoplasmic locus each with 2 alleles, maternal inheritance of the cytoplasm, an

infinite population size, no homoplasy, no overlapping of generations, and sufficient pollen to maintain the population.

According to this model, conditions involved in chloroplast capture include:

- A higher female fitness in the genotype that has the invading cytoplasm with the resident nuclear alleles compared to the genotype with both the resident cytoplasm and nuclear alleles.
- A lower fitness of the heterozygotes with the resident cytoplasm compared to the fitness of the resident homozygotes with the invader cytoplasm.
- A lower fitness of the heterozygotes with the invading cytoplasm compared to the fitness of the resident homozygotes with the invading cytoplasm.

These conditions favor the production of the homozygotes of the resident nuclear alleles with the invading chloroplast, a condition that indicates successful chloroplast capture. However, if a certain percentage of nuclear genes introgress along with the invading chloroplasts to the resident species, conditions would be less restrained.

Theoretical introgression rates have been calculated to occur in about 1000 generations. Actual experimental data of introgression rates of *H. annuus* cytoplasm into *H. petiolares* has been documented to occur in less than 50 generations. In addition, selfing rates of populations can play an important role in introgression rates. Reduction of the selfing rate as a result of genome incompatibilities is predicted to increase the rate of chloroplast capture. If, on the

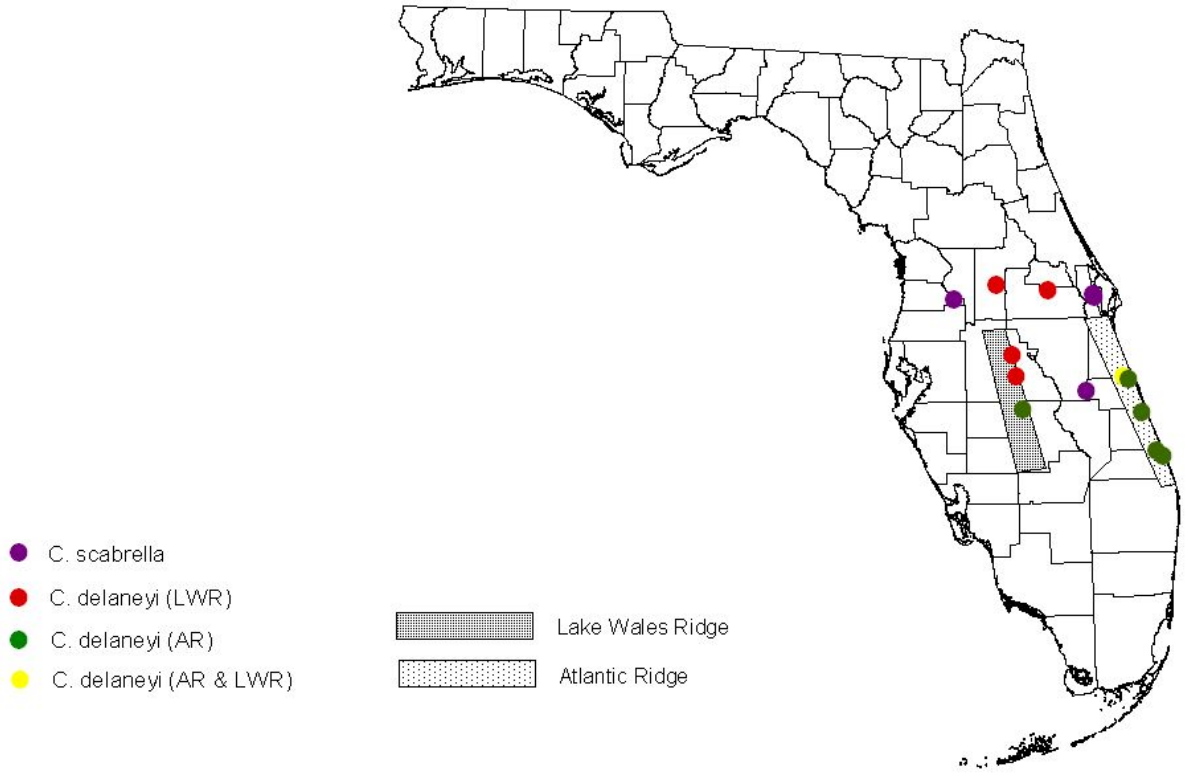
other hand, the selfing rate of the resident is not affected by these incompatibilities, the rate of chloroplast capture would be similar to a randomly mating population (Tsitrone *et al*., 2003).

In order to use this model as a basis for chloroplast capture events in populations of *C. delaneyi* the assumptions must be examined. First, the chloroplasts are maternally inherited in the species. Next, there appears to be an adequate supply of pollen to maintain natural populations and, in addition, it has been estimated by comparison studies with similar species that approximately 5-10% of selfing may occur in this species (Semple, personal communication). Furthermore, the prevalence of homoplasy in these different populations of *C. delaneyi* does not seem feasible because the probability of these closely related species undergoing unrelated mutations that result in the same character state as compared to inheriting the character state appears unlikely. However, populations of *C. delaneyi* are not of infinite size and in particular, LWR populations are more reduced compared to AR populations. These plants are short-lived perennials, therefore this may violate the assumption of no overlapping of generations.

Populations of *C. scabrella* are found throughout Florida. Samples used in this study come from populations located in Hernando, Brevard, and Osceola Counties. The LWR samples were taken from Lake, Orange, and Polk Counties. The LWR populations are centrally located within the outlying *C. scabrella* groups. The exception is the Osceola County population of *C. scabrella*. This particular group is situated closer to AR populations. Populations of *C.*

highlandsensis are found in Polk County as well, but also in central southern Highlands and Glades Counties. AR populations occupy counties along the eastern coastline, starting from Indian River south to Palm Beach (Figure 7). These spatial patterns demonstrate the proximities of *C. highlandsensis* and AR populations, as well as LWR and *C. scabrella*.

Figure 7: Population Distribution Map of *Chrysopsis* species



Introgression appears to be a plausible cause for the discrepancies found within the chloroplast genome of *C. delaneyi* populations. Although extant populations are allopatric to *C. scabrella* and *C. highlandsensis* populations, speculation of historic spatial patterning could include more contiguous ranges. Groups that have a tendency to hybridize may eventually replace one of the species, however until replacement is completed, the population may demonstrate features of being mixed or parapatric (McKinnon *et al.*, 2004). As in the case of *C. delaneyi*, two such populations do exist. First is the mixed haplotype population at the IRCL location, and the second is at RSLND, a population located directly southeast of IRCL, which contain 2 distinct haplotypes, one from each of the AR and the LWR haplotype. Adaptive strategies to the different landscaping and environmental conditions of Florida may have influenced the morphological changes found within *C. delaneyi* species. AR populations, as previously indicated, are larger plants with a more sturdy structure than are the LWR populations. These populations, which are established in open, sandy areas, may have adapted both physically and genetically to be more conducive to such harsh conditions. Accordingly, adaptation to the shaded turkey oak sandhills and longleaf pine habitats may have contributed to the reduction in plant size of LWR populations.

Alternative hypotheses to introgression have been described in several papers, including Comes *et al.* (1997), Tsitrone *et al.* (2003), McKinnon *et al.* (2004), and Okuyama *et al.* (2005). The first of these is lineage sorting. This process involves either the preservation or elimination of ancestral

polymorphisms in the descendant groups. This symplesiomorphic condition between AR and *C. highlandsensis* is not very apparent. A more convincing scenario for this hypothesis would be that the polymorphisms would be shared within AR and LWR *C. delaneyi* and possibly *C. scabrella*, as these groups are more closely related to each other than they are to the more distantly related *C. highlandsensis*. The next hypothesis is that of convergent evolution. This reflects a condition of homoplasy rather than identity by descent. Although the populations of *C. highlandsensis* and AR are geographically close to each other, the probability that both have undergone similar mutation processes as a result of adaptation to similar environmental conditions resulting in the same shared polymorphisms appear to be coincidental. The same can be said for *C. scabrella* and LWR populations. Finally, recurrent hybridization has been suspect with inconsistencies found in gene trees. This process involves frequent hybridization events that would affect either the organellar or nuclear genomes, which in turn would be passed on in a directional pattern to a resident species. While these alternative hypotheses seem unlikely in *C. delaneyi* populations, further testing is needed to completely rule these out.

The most likely status for the ancestral state, based on the chloroplast data, appears to be that found in the *C. highlandsensis* and AR *C. delaneyi,* and the LWR *C. delaneyi* and *C. scabrella* are derived from this character state. While the restriction enzyme digests, documented to be identical for restriction enzymes in closely related species (Olmstead *et al.*, 1994), lend support to the unconventional relationships between these groups by either the loss or the

acquisition of both restriction sites, the most convincing evidence comes from the direct sequencing of the ccmp3 region, which uncovered 2 insertion sites in the latter two groups. In addition, the alignment from the BLAST search of 7 plant families, including 3 Asteraceae, was used to assist in the determination of the ancestral state. The 4 bps that aligned in the first insertion site were not clearly comparable to those of the sample sequences. The alignment of the 18 – 21 bases of the outgroups at the second insertion site show no signs of a duplication event as was found in the study samples. Additionally, these bps are exact in the 3 Asteraceae families and similar with the exception of 1 bp difference in the Solanaceae and Altingiaceae groups. The Rosaceae and Caryophyllaceae groups are both distinct from all of the others. Comparisons between and within these families contribute to the conclusion that *C. highlandsensis* and AR contain the ancestral state. Nuclear data will be required to verify the ancestral state because the nature of inheritance of the chloroplast genome as a complete single unit may interfere with interpreting patterns of species divergence due to introgression events (Olmstead *et al*, 1994).

CONCLUSION

The relationship between the LWR and AR C. delaneyi populations has not been resolved to its fullest potential. Clearly a distinction between *C. delaneyi* species has been identified based on chloroplast data alone, which may be the result of chloroplast capture. The discovery of the distinct haplotypes gives rise for the need to distinguish suitable conservation management practices for each individual ecotype if these populations are to be maintained in the wild. The LWR populations seem to be more at risk of eventual extinction than the AR populations. Although these populations have not yet been listed as endangered, factors such as human encroachment and recent years of severe weather conditions have proven detrimental to existing populations. Unless plans are implemented soon, these plants are at severe risk.

In order to carry out a sound conservation program, proper identification and prioritization of species, knowledge of habitat requirements as well as genetic diversity in populations must be established (Partel *et al.*, 2004; Lee *et al.*, 2006). The viability of a population is controlled by its vital rates, which in turn are affected by genetic and environmental processes, respectively. An increase in genetic diversity may be a key component in a population's ability to survive environmental changes, either natural or anthropogenic (Lee *et al.*, 2006). With an increase of human influx into sensitive areas, habitat fragmentation is

increasing. Once plant populations become isolated, the general trend is a decrease in genetic diversity and a decrease in population fitness. Therefore, factors that need to be considered when designing a conservation plan should include the size of the population and a method to strengthen gene flow among populations (Gao, 2005).

One possible solution to prevent the loss of genetic diversity would be to generate a seed bank and stock plant collection of the wild plant populations. These collections must be representative of the actual population in order to maintain the genetic integrity of the wild populations if it becomes necessary to use the seeds or plant stocks for restoration purposes. If care is not taken when establishing a seed bank and the seeds are used in the wild populations, the genetic structure will be altered. Other options of conservation may want to be considered first. However, if these populations are already severely isolated and lack genetic diversity they may require an influx from other populations in order to increase their fitness (Segarra-Moragues *et al.*, 2005).

With regards to the *C. delaneyi* populations, it is clear that two separate management programs would be needed. The LWR populations are more fragmented and isolated than the AR populations. In addition, there appears to be little chloroplast divergence between the LWR and *C. scabrella* populations. The AR populations are, for the time being, more robust and less fragmented than the LWR populations. Therefore, they may not have been subjected to a loss of genetic diversity as a result of isolation, thus maintaining the ancestral state of genetic variation. Therefore, conservation management would want to

include these larger populations in their conservation efforts in order to preserve the ancestral structure of the species. In concert with this logic, allopatric populations such as the LWR populations demonstrate the change of genetic structure of the species as a result of adaptation processes thus representing important components in the evolutionary history of a species warranting protection as well. Within population variation like those found in *C. scabrella* populations results when the absence of gene flow from other populations occur after events such as Founders Effect or genetic drift. Natural selection takes over, selecting the genotypes that are most fit for the conditions of the establishing species. Again, this process warrants protection of species in order to maintain intraspecific variation by maintaining the gene flow among populations (Gao, 2005).

The use of chloroplast molecular markers has proven effective in detecting cpDNA variations in this study. They have identified two haplotypes of *C. delaneyi*, one haplotype within a population of *C. scabrella*, and one haplotype within an AR population. Nuclear data is needed to corroborate the results. The nuclear markers used in this study were not as effective, thus optimizing conditions for these reactions will be required in order to produce accurate data.

FUTURE DIRECTION

As stated in the definition of an ESU by Moritz (1994), data from the nuclear genome must be examined along with the organellar genome and show a significant amount of divergence in order to accurately determine phylogenies of species, and determine an ESU. Therefore, more progress will need to be made with the nuclear genome. Initial reactions conducted in this study that involved PCR amplification and sequencing of the intron region of the Actin 1 gene proved to be problematic. One solution would be to design primers for this region either from sequences retrieved from successful reactions on the *C. delaneyi* samples or by searching for similar sequences in the Asteraceae family using Genbank. Additionally, exploring other nuclear regions such as the glycerol-3-phosphate acyltransferase (GPAT) gene (Tank & Sang, 2001), the nitrate reductase intron (Howarth & Baum, 2002), and the glyceraldehyde 3-phosphate dehydrogenase (G3pdh) gene (Strand *et al.*, 1997) may prove more successful.

The cpDNA variations in natural populations need to be monitored in order to evaluate the amount of genetic diversity maintained in populations. Initial data collected serves as a baseline of the chloroplast genomic structure of these populations at the current time. This aspect is important for the mixed IRCL

populations and RSLND populations in order to determine current evolutionary and adaptive processes taking place.

To investigate the introgression hypothesis, experimental design of plant crosses would determine the female fitness requirements proposed by Tsitrone *et al.* (2003). For example, setting up crosses between *C. highlandsensis* and AR *C. delaneyi,* using ovules from *C. highlandsensis* and the pollen from a close relative of AR *C. delaneyi* that does not contain the invading chloroplast. A cytoplasm substitution line would be created by repeated backcrossings and the female fitness could be determined by seed production. Along with chloroplast markers, mitochondrial markers can be used to compare both of these gene trees together. Similarities among them would indicate that introgression of the chloroplast has occurred. If, however, there are inconsistencies found, homoplasy may be involved.

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