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Analysis of microsatellites from Sclerocactus glaucus and Sclerocactus parviflorus to assess hybridization levels and genetic diversity

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ANALYSIS OF MICROSATELLITES FROM *SCLEROCACTUS GLAUCUS* AND *SCLEROCACTUS PARVIFLORUS* TO ASSESS HYBRIDIZATION LEVELS AND GENETIC DIVERSITY

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ABSTRACT

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Sclerocactus glaucus is an endemic Colorado species that is federally threatened under the Endangered Species Act. *Sclerocactus glaucus* is losing habitat due to disturbance by oil and gas exploration, urbanization, open range cattle grazing and recreational land use. Due to the low number of wild populations, conservationists question the genetic integrity of the species. Field biologists have observed *S. glaucus* populations with individuals possessing morphological characteristics of the closely related and widely distributed *Sclerocactus parviflorus.* Individuals from 28 populations of *S. glaucus,* 9 populations of *S. parviflorus*, and 1 population of *S. cloveriae* were sampled. Microsatellite analysis using 13 variable loci was used to determine population structure, degree of hybridization, gene flow, and diversity levels of these species. Chloroplast DNA analysis was also used to determine diversity, phylogenetic relationships, and direction of gene flow. Using genetic tools, the analyses established that *S. glaucus* populations remain diverse and mostly untainted by hybridization. These data also demonstrate that morphology is not reliable for identification of species or hybrids within this cluster of species. Characters that historically designated *S. parviflorus,* such as hooked spines, were found not to be good indicators for species

determination. Two populations of *S. glaucus* were misidentified as *S. parviflorus* and one of these populations is a genetically pure population with no genetic introgression from *S. parviflorus.* Species divisions appear to be closely tied to geographical location with *S. parviflorus* located only to the east of Grand Junction. Two distinct groups of *S. glaucus* are distinguished by the river drainage systems in which they are located. Land managers and conservationists now have the genetic information to move forward with preserving populations of *S. glaucus*.

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CHAPTER I

REVIEW OF THE LITERATURE

Introduction

This research project is a genetic investigation of the federally threatened *Sclerocactus glaucus* (K. Schumann) L.D. Benson (Cactaceae), commonly known as the Colorado hookless cactus. *Sclerocactus glaucus* is found in small populations in western Colorado, on rocky slopes and lowland mesas around Grand Junction (U.S. Fish and Wildlife Service [USFWS] 2010). *Sclerocactus* populations are being depleted by disturbance from oil and gas exploration, urbanization, trampling from livestock, disease, predation, off road vehicle damage, and over-collecting (U.S. Fish and Wildlife Service [USFWS] 2007). While human activities are affecting *S. glaucus* numbers, hybridization with a common relative is also a cause of concern among conservationists. This project was developed to examine the genetic structure within and among populations, and explore the potential threat of gene flow from the closely related common congener, *Sclerocactus parviflorus* Clover and Jotter. The knowledge gained through these analyses will allow us to understand how *Sclerocactus* species interact, and add an evolutionary dimension to *Sclerocactus* conservation. The genetic information in this study will assist in defining which populations might be considered for conservation priority.

The goal for the project was to collect data from nuclear microsatellites and chloroplast DNA sequence markers to determine the level of diversity within and among *S. glaucus* populations as well as the level of hybridization between *S. glaucus* and *S.*

parviflorus. Previous research on *Sclerocactus* is limited and has involved some morphological character analysis, chloroplast genome analysis, amplified fragment length polymorphism (AFLP) analysis and common garden hybridization experiments (Porter et al. 2000; Porter et al. 2007; Tepedino et al. 2010). However, genetic studies that have used microsatellites to assist conservation efforts are common for rare and endangered plant species. Variation at microsatellite loci has been used to determine geographic distributions of species, population genetic structure, genetic diversity, hybridization, populations of interest for conservation, parentage, and pollen and seed dispersal (Anderson and Thompson 2001; Ashley 2010; Gao and Zhang 2005; Petit et al. 1997; Spruell et al. 2003; Viana e Souza and Lovato 2010). Genetic analysis gave insight as to which populations of *S. glaucus* had little or no introgression from *S. parviflorus.* Using both nuclear microsatellite markers and chloroplast DNA allowed genetic resolution of gene flow between the species, which will assist in making land management decisions. If diverse populations of *S. glaucus* exist with minimal or no gene flow from *S. parviflorus*, they should be given conservation priority.

DNA samples obtained from 865 individuals in 38 populations were analyzed with 13 variable microsatellite loci. The data were used to analyze structure and gene flow within and between populations. Hybrid populations as well as hybrid individuals were pinpointed, and the extent of introgression into populations of *S. glaucus* was assessed. Chloroplast DNA analyses were also carried out with data from two intergenic spacers, trnF-trnL and trnC-rpoB, for hybrid or genetically unique individuals from many populations, to determine the species of chloroplast origin. This analysis was done to give a sense of the directionality of hybridization.

The data generated from this study provide information about genetic relationships among populations of *S. glaucus* and *S. parviflorus*. These relationships included levels of gene flow not only between *S. glaucus* and *S. parviflorus* but also between populations of *S. glaucus.* The genetic diversity of each population was examined and populations of interest, such as pure or populations with unique diversity, were determined and suggestions were made for conservation priority. The data from these analyses can be used by conservation managers to make land management and species recovery decisions (USFWS 2007). The recovery outline for *S. glaucus* from the USFWS Recovery Plan recommends increasing the priority ranking from 14C, which is a low degree of threat, to 8C, which is a moderate degree of threat (USFWS 2010). The Recovery Plan recognizes *S. glaucus* as a distinct species with a moderate degree of threat, a high potential for recovery and is in conflict with development and/or economic activities (USFWS 2010). Research for the initial action plan for the recovery plan includes resolving the taxonomic status with regards to the relationship between *S. glaucus* and *S. parviflorus* (USFWS 2010). The plan also calls for a genetic assessment of the differences among *S. glaucus* populations. Finally, using genetic tools, population dynamics and population vulnerability can be assessed and used in initial action plan for the recovery plan (USFWS 2010).

Sclerocactus **Genus**

The genus *Sclerocactus* was first described in the early 20th Century (Britton and Rose, 1923) and originally including two species. Today, *Sclerocactus* has grown to include 15 species (Heil and Porter 2004). Historically, *Sclerocactus* species were identified based on morphological characteristics such as spine morphology, size and

seed coat variations (Hochstätter 1989; Porter et al. 2007). These morphological characters have been found to be highly plastic not only between species, but also within taxa (Porter et al. 2000). *Sclerocactus glaucus* was listed under the Endangered Species Act (ESA) on October 11, 1979 (U.S. Fish and Wildlife Service 1979). The USFWS officially split *S. glaucus* into three separate taxa; *S. glaucus* (the Colorado hookless cactus), *S. brevispinus* (Pariette cactus) and *S. wetlandicus* (Uintah hookless cactus) on September 15, 2009 (USFWS 2010). All three species are protected under the ESA.

The taxonomy of *S. glaucus* populations has been described as being one of the most confused in the genus (Porter et al. 2007). Historical descriptions and collection records indicate that *S. glaucus* occurred in two disjunct areas, western Colorado and northeast Utah, but recently it has been segregated into three distinct taxa, *S. glaucus, S. brevispinus* and *S. wetlandicus* (Heil and Porter 2004; USFWS 2007). *Sclerocactus glaucus* is distinguished from *S. brevispinus* and *S. wetlandicus* by seed coat micromorphology and geographical location (Heil and Porter 2004; Hochstätter 1989). *Sclerocactus glaucus* has convex cells on the seed coat surface while *S. brevispinus* and *S. wetlandicus* have flat cells on the seed surface. The geographical range of the newly recognized *S. glaucus* is confined to Colorado and has not been described beyond the Colorado border, while the other two taxa are located in northeast Utah (Heil and Porter 2004). Both micromorphology of the seed coat and geographical location are used to determine species but these three species have multiple shared morphological characters that make them difficult to distinguish from one another (Porter et al. 2007)

Life History

Sclerocactus glaucus is traditionally identified in the field by the absence of hooks on the spines along with geographical location and to some extent, size and flower color. The size of the individual plants could be related to age and/or the quality of the habitat (USFWS 2010). The life cycle, development and longevity of *S. glaucus* are largely unknown. Demographic long-term monitoring of some populations by the Denver Botanic Gardens has begun but has not been established long enough to gain accurate details relating to how long-lived the species is. Additionally, little is known about the pollinators and modes of dispersal are largely unknown. A pollinator study by Tepedino et al. (2010) in Utah on *S. brevispinus* and *S. wetlandicus* has revealed that the two closely related species are pollinated by native bees. Since the species are closely related, assumptions can be made that *S. glaucus* is more than likely pollinated by native bees also. Other assemblages of insects including beetles and ants may be involved in cross-pollination as well (USFWS 2010).

Morphology

Sclerocactus glaucus, S. brevispinus and *S. wetlandicus* are relatively small barrel shaped cacti, 3-12cm high and 4-9cm in diameter. *Sclerocactus wetlandicus* is often found to be much larger than either *S. glaucus* or *S. brevispinus* (Heil and Porter 2004). The barrel of the cactus has 8 to 15 ribs that extend along the entire stem and 1-5 spines per areole (Heil and Porter 2004). All three species have funnel shaped flowers with pink to violet inner tepals, similar fruits, which are indehiscent oval shaped berries, and black seeds (Heil and Porter 2004). Although taxonomic descriptions for *S. glaucus* and other *Sclerocactus* species have historically been made on the previously mentioned

characteristics, there is high morphological variation at the species level (Porter et al. 2007). Polymorphic characters potentially become even less reliable when attempting to identify hybrid individuals and hybrid populations of *Sclerocactus* species.

Spine morphology was previously thought to have been a dependable character to differentiate between *S. glaucus* and *S. parviflorus* (Porter et al. 2007). Traditionally straight spines have been associated with *S. glaucus,* while hooked spines are a discerning characteristic of *S. parviflorus*. These characteristics have been found to be highly variable, with populations of *S. glaucus* displaying both hooked and straight spines. While some populations include hooked individuals and hook-less individuals, there are also individuals with mixed morphologies. It has been suggested that individuals with both or intermediate spine types, may be morphologically indicative of hybrid individuals. Although known populations of *S. glaucus* are protected under the Endangered Species Act, the actual number of individuals may not be accurate if populations have been misidentified.

Previous Phylogentic Work

In addition to the already confusing morphological taxonomy of *Sclerocactus* species, a phylogenetic study conducted by Porter et al. (2000) found that the evolutionary history of *Sclerocactus* is unresolved. Five currently recognized species of *Sclerocactus* (*S. glaucus, S. parviflorus, S. brevispinus, S. wetlandicus* and *S. cloveriae*) fall out together in an unresolved clade (Porter et al. 2000). This phylogenetic research was done using slowly evolving chloroplast DNA, which would not necessarily reflect recent speciation. The poor resolution from the chloroplast data indicates a need for additional work using a higher number of variable markers. Many of the branches on

phylogenetic trees from this chloroplast study were unresolved. A study conducted by Porter et al. (2007) using AFLP markers on *S. glaucus, S. brevispinus* and *S. wetlandicus* concluded that *S. glaucus* has diverged significantly from the individuals found in Utah. However, financial limitations allowed only a small number of *S. glaucus*, *S. brevispinus*, and *S. wetlandicus* to be analyzed and the researchers recognized that this study was preliminary (Porter et al. 2007). Chloroplast data was inconclusive while AFLP resolved some distinctions between *S. glaucus* in Colorado and *S. brevispinus* and *S. wetlandicus* in Utah (Porter et al. 2000; Porter et al. 2007). The taxonomic divisions of *S. parviflorus* and *S. glaucus* have not previously been analyzed with any resolution as to whether or not they are distinct and separate species.

The time since divergence of *Sclerocactus* species is unclear, and for this reason, a more rapidly mutable section of the genome could be more informative when attempting to clarify taxonomy within this group. Microsatellites, AFLPs and allozymes are more rapidly evolving and can provide information about more recent events, but may be too mutable to provide useful information about ancient speciation events (Porter et al. 2007). Phylogenetic relationships may become clear after examination of nuclear microsatellite regions and studying population genetic structure. The results can then be applied to morphological variation across populations as well as the location of species and populations in geographical space to clarify some of the concerns related to *S. glaucus* and *S. parviflorus*.

The morphological inconsistencies within and among species have uncovered the need for a more in-depth genetic investigation to determine if there are hybrid individuals and/or hybrid populations. If there are hybrid populations, the level of introgression of

the *S. parviflorus* genome into *S. glaucus* populations would need to be assessed (Porter et al. 2000; Porter et al. 2007). For the most effective conservation plan, the correct species identification needs to be determined from genetic analyses as well as establishing the distribution of each taxon in order to assign conservation priority.

Species Definitions

Recently, conservation biologists have become concerned that *S. glaucus* and *S. parviflorus* are hybridizing due to potentially overlapping ranges and observations of hooked spines within populations previously identified as *S. glaucus.* Hybridization concerns arise when rare species in small populations are exposed to a potentially large influx of genetic material from a closely related species (Rhymer and Simberloff 1996). Gene flow from *S. parviflorus* into small populations of *S. glaucus* could possibly overpower and eradicate the *S. glaucus* genome. If there is a high degree of introgression with many *S. glaucus* hybrids within populations, over time, this may effectively render *S. glaucus* extinct as a direct result of genetic dilution of the *S. glaucus* genome. Hybridization occurs naturally and is thought to be one of the driving forces of speciation (Coyne and Orr 2004). Range contraction from natural disturbances or environmental changes can isolate small pockets of individuals (Ellstrand and Elam 1993). Over time these isolated populations will experience different selection pressures and diverge due to genetic drift (Ellstrand and Elam 1993). The separated populations will gain and lose various alleles due to genetic drift, random mutation, and or local adaptation. Over time genetic divergence of the isolated populations can result in two different groups, which, if different enough, can be described as two different species (Ellstrand and Elam 1993).

The most widely accepted idea of distinguishing a species is the Biological Species Concept (BSC) (Mayr 1995). This conceptualization of identifying distinct species is based on the ability for individuals to interbreed. Mayr (1995) states "species are groups of interbreeding natural populations that are reproductively isolated from other such groups." Reproductive isolation may be a physical separation where gene flow is cut off by either a barrier or physical distance. Isolation may also be due to extensive genetic divergence resulting in reproductive incompatibility. If the genome has become different over time then reproduction between the two previously connected populations is no longer possible. Conversely, expansion can bring formally isolated populations into contact again. Depending on the degree of isolation and genetic divergence of populations from each other, they may or may not still be reproductively compatible and able produce viable offspring. According to the BSC, populations that come into contact, reproduce and produce viable offspring, would not be considered distinct species. The BSC has been applied to many organisms in the animal kingdom, but is not necessarily appropriate to apply to plants since many related plant species readily hybridize (Rieseberg and Carney 1998). Strict application of the BSC would propose that if two species of *Sclerocactus* had overlapping ranges and were able to hybridize, then the two species would be considered a single species. This approach is not applicable if genetic work clearly identifies separate species in a particular genus even if there are a few hybrid individuals, which is common in plant populations.

Although the BSC may not work well for plant species, there are many alternative species concepts, which may be more applicable. Species concepts revolving around evolutionary histories, which use phylogenetic relationships to identify species, seem to

fit better when dealing with plants. Cracraft (1989) describes species in a phylogenetic manner as "an irreducible (basal) cluster of organisms that is diagnosably distinct from other such clusters, and within which there is a paternal pattern of ancestry and decent." De Queiroz and Donoghue (1988) describe a species as "the smallest [exclusive] monophyletic group of common ancestry". These concepts allow speciation to be dictated and supported by genetics and could be applicable to most living organisms.

Using genetic analysis it is possible to discern diagnosably distinct groups as well as patterns of descent, as Cracraft (1989) suggests. The genetic data can also be used to determine phylogeny and monophyly as de Queiroz and Donoghue (1988) recommend. Applying these last two concepts to *Sclerocactus* takes into consideration that *S. glaucus* and *S. parviflorus* are not necessarily reproductively isolated and therefore have the ability to hybridize in natural populations. If there is extensive hybridization between these species, a reticulation event, or combining of the two lineages into one, may be cause for concern. Continued genetic work will bring to light whether *Sclerocactus* species are hybridizing and if the possibility of a reticulation event is possible.

Conservation

Molecular markers can be useful in determining if specific populations of rare species should be targeted for management (Spruell et al. 2003). Conservation biologists attempt to preserve or restore species that have undergone a loss in numbers due to habitat loss, exploitation or environmental change. Population geneticists analyze gene frequencies under the influences of drift, selection, mutation and gene flow, and attempt to explain adaptation and speciation using genetic information from populations. Using tools from both of these fields, it may be possible to determine the underlying genetic

processes, such as hybridization and gene flow, responsible for shaping species and use the information to make informed management decisions. Distinctiveness and diversity are two important factors in making these decisions (Barrett and Kohn 1991; Gonzalez-Perez et al. 2009; Viana e Souza and Lavato 2009). When the genetic structure of populations is uncovered and Evolutionary Significant Units (ESU) are identified, the best management strategy can be implemented. The concept of ESUs was developed to prioritize distinct taxa or populations for conservation (Moritz 1994). With this information land managers may enforce boundaries for habitat protection, remove and transplant unique populations to a protected area, or perhaps set up monitoring of populations to ensure persistence.

Sclerocactus glaucus is located in two population centers on alluvial terraces of the Gunnison River, and of the eastern Grand Valley and Colorado River drainages. (USFWS 2010). Porter et al. (2007) suggested that these areas could contain unique and distinct populations of *S. glaucus* that are genetically discrete from each other and these differences may be due, in part, to introgression from *S. parviflorus*. The Colorado National Heritage Program (CNHP 2010) has reported 98 Element Occurrences (EO) of *S. glaucus* containing approximately 13,000 individuals (USFWS 2010). Of the 98 EOs described by the CNHP (2010), 42 have not been observed in over 20 years (USFWS 2010). The Natural Heritage Network uses the term element occurrence to describe a basic conservation unit and is an area where a species is or was present and has practical conservation value (CNHP 2005). Multiple EOs may be assigned to a single population when a population is large and multiple observed occurrences span a large population. It is relatively common to have an increased number of EOs compared to the number of

actual populations. Therefore, the number of EOs may not be an accurate indicator of the number of populations of a species (M. McGlaughlin, personal communication). In order be considered an element occurrence of *S. glaucus*, the individual or population must be located in an appropriate habitat and in a natural community (CNHP 2005). With less than 100 EOs, in addition to its limited range and widespread threats, *S. glaucus*, has a vulnerable global imperilment ranking (NatureServe 2012). The global imperilment rank is based on the described number of EOs characterizes the rarity or endangerment of the species worldwide (CNHP 2005). Project surveys by Bio-Logic have uncovered more than 6,000 individuals that have not previously been described and have not yet been added to the CNHPs database (USFWS 2010). These newly discovered populations of *S. glaucus* put estimated numbers of individuals at over 19,000 (USFWS 2010). *Sclerocactus parviflorus* has a range of 21-80 EO's, which would place it in the vulnerable global imperilment ranking (NatureServe 2012). However, due to the large number of individuals, lack of *S. parviflorus* specific monitoring, and large range that populations cover, *S. parviflorus* is currently globally ranked as apparently secure (NatureServe 2012).

Sclerocactus glaucus are very difficult to locate in the field due to their small size and color. They are most noticeable in the short time when they are in flower and much of the known potential habitat has not been surveyed (USFWS 2010). *Sclerocactus glaucus* occupies a range spanning 1,700 square miles with only 618,000 acres of possible habitat and of that the available habitat, ~28%, is on land where the plants would receive little to no protection, such as private lands (USFWS 2010). Conservationists are concerned about land developments in the area and that recovery efforts of *S. glaucus*

may require either transplanting or destroying up to 100 individuals (USFWS 2010). If transplanting individuals or entire populations is an option, understanding genetic relationships, structure, distribution, hybridization and diversity is needed. Transplanting misidentified individuals or hybrid populations could have diverse effects on previously established populations.

The possibility that human activities are promoting hybridization between *Sclerocactus* species has gained attention from conservation biologists (Tepedino et al. 2010). Although some gene flow between species is considered normal, corridors between populations created by human activities could be problematic for the continued existence of *S. glaucus* (Anderson 1948; Rieseberg and Carney 1998). Human activities may have led to the breakdown of isolating barriers and without isolating barriers gene flow increases and gene pools are mixed, leading to loss of genetically distinct populations (Rhymer and Simberloff 1996). Conservation biologists and land managers have the task of protecting *S. glaucus* from further impacts by human activities. Questions that surround *S. glaucus* need to be clarified before land managers can effectively tackle the continued preservation of *S. glaucus* and its habitat.

The USFWS recovery plan for *S. glaucus* begins with recognition of *S. glaucus* as a distinct species. In order to move forward then, it is necessary to definitively determine through genetic analysis if in fact *S. glaucus* and *S. parviflorus* are distinct and separate species. Initial phylogenetic work conducted by Porter et al. (2000) using chloroplast DNA was inconclusive regarding distinct *Sclerocactus* species. However, due to the intermediate morphologies that have been observed in various populations, it is possible that some populations have been misidentified or represent hybrid swarms. Correctly

assigning species to populations is important so that conservation efforts are directed at protecting the endangered *S. glaucus* and not protecting a misidentified population of *S. parviflorus*. Conversely, if a population has been identified as *S. parviflorus* and it is actually a population of *S. glaucus*, conservation steps need to be taken to preserve those individuals. Next, levels of hybridization need to be assessed (Wan et al. 2004). Land managers should target for conservation populations of *S. glaucus* that are found to have no introgression or minimal gene flow from *S. parviflorus*. Populations with a high level of introgression from *S. parviflorus* may be given a lower priority for conservation. Finally, there may be populations of *S. glaucus* that contain high levels of or unique genetic diversity that may be of importance when considering the future existence of the species.

Data collected in this study will help to clarify many of the conservation issues surrounding *S. glaucus*. First of all it will give additional support to the idea that *S. glaucus* and *S. parviflorus* are separate and distinct species. If *Sclerocactus* species have recently split then a rapidly mutating genetic tool, such as microsatellite analysis, should be used to reflect evolutionary patterns in the genus. Therefore using microsatellite data from the nuclear genome may help clarify some of the taxonomic and phylogenetic questions surrounding this genus. Patterns of gene flow will be examined to determine how these species are interacting, both among *S. gluacus* and *S. parviflorus* as well as among *S gluacus* populations. *Sclerocactus glaucus* populations are arranged in two separate drainages that merge in Grand Junction. The northern populations are in the Colorado River drainage near De Beque. The southern populations are in the Gunnison river drainage near Delta. Uncovering vital genetic information will help define

populations for conservation priority, expand our understanding of species interactions within *Sclerocactus,* and add an evolutionary dimension to conservation activities.

Overview of Content

The chapters that follow contain the methods used to retrieve variable microsatellite and chloroplast DNA (cpDNA) data as well as the statistical analyses leading to conclusions and suggestions for conservation of *S. glaucus.* Chapter II details the methods and protocols used in this genetic study. It includes a description of DNA extraction procedures designed for this project and microsatellite marker design. Chapter III includes the extensive microsatellite research that will be used to help make management decisions to conserve this rare Colorado plant. It contains the methods used for this study and the statistical analyses from the data gathered. The results are then interpreted and discussed, which will help direct conservation efforts. Chapter IV is an analysis of the chloroplast genome from pure and putative hybrid individuals in *S. glaucus* and *S. parviflorus*. These data are analyzed to determine directionality of the hybridization and some biogeographical inferences. Chapter V is the final chapter, which summarizes the contents, presents a synopsis of the results and concludes the findings of the investigation.

CHAPTER II

METHODS AND PROTOCOLS DESIGNED FOR THE ANALYSIS OF *SCLEROCACTUS* DNA

Introduction

The methods and protocols outlined in this chapter are DNA extraction and microsatellite marker design. The DNA extraction protocol was modified because cactus tissue contains high amounts of polysaccharides and other secondary compounds that make DNA extraction difficult with traditional methods. Without the modifications contained herein, subsequent PCR amplifications of the variable loci would not be successful and would ultimately yield a poor data set. The microsatellite marker design is excerpted directly from published data (Schwabe et al. 2012).

Nuclear microsatellite markers are frequently used to analyze genetic composition of populations (Morgante and Oliveri 1993). Microsatellites are regions of DNA containing simple sequences of short repeating nucleotides (2-4 bases) (Braaten et al. 1998; Hamada et al. 1982; Schafer et al. 1986; Tautz and Renz 1984; Vergnaud 1989). Microsatellite regions occur frequently in the genomes of all eukaryotic organisms, are easily identifiable, and are considered to be hypervariable (Morgante and Oliveri 1993). Due to the variability of microsatellites, they can be used as markers to compare individuals within and among species and populations (Morgante and Oliveri 1993). Variation in individuals across multiple loci can be used to determine diversity within a species (heterozygosity), as well as levels of inbreeding (F_{IS}), hybridization, degree and

direction of gene flow, effective population size (N_e) as well as the genetic structure of populations (Guichoux et al. 2011).

DNA Extraction Procedure

DNA from 885 individuals from 38 populations was extracted using a modified version of the DNeasy Plant Mini DNA extraction kit (Qiagen) protocol. The protocol is was modified specifically for *Sclerocactus glaucus* and *S. parviflorus* but has been successful in DNA extraction for other *Sclerocactus* as well as *Ficus elastica* (Moraceae), *Kalanchoe daigremontiana* (Crassulaceae), *Hibiscus* sp. (Malvaceae), and *Schlumbergera* sp. (Cactaceae) plants which have previously shown poor DNA extraction results.

Floral bud tissue was used for the DNA extraction procedure. Floral tissue is preferred for rare cactus species, as most cacti do not have leaves. Although stem tissue is available, taking samples from the barrel may damage the plant or expose it to disease. Flowers are only taken from plants with more than one bud so reproduction can continue. The floral tissue was stored in silica gel, ensuring complete dehydration of the samples. This was beneficial for preserving the DNA by dehydrating proteins, enzymes and secondary metabolites. Silica gel also eliminated the need for refrigeration of specimens and makes the grinding process easier. The Plant Tissue (Mini Protocol) from the July 2006 edition of the Qiagen DNeasy Plant Handbook was used (pp. 24-27) with the modifications detailed below.

The initial mechanical lysis of the cell wall was achieved in steps 1-6 of the Qiagen protocol. These steps were modified for *Sclerocactus* DNA extraction. A small amount of desiccated floral bud tissue was ground using liquid nitrogen and a clean

mortar and pestle. The ground tissue was then put into a 1.5 ml microcentrifuge tube. The amount of tissue was not to exceed the 75µl mark on the tubule. Some of the samples were small, so a maximum of one half of the available tissue was used for extraction. Step 7 of the Qiagen protocol was further lysing of the cell membranes and exposing the DNA by adding 800 µl of AP1 buffer and digestion of RNA by adding 4 µl RNaseA. An additional step was added for the *Sclerocactus* DNA extraction. After adding AP1 buffer and RNaseA to each tube and vortexing, additional AP1 buffer, up to 700 µl was added to the solution if the lysate was too viscous. Viscosity was determined visually after vortexing by inverting the tube and observing the mixture's thickness. If the consistency of the liquid was more viscous than oil, additional AP1 buffer was added. A sterile pipette tip was used to remove any tissue that remained at the bottom of the tube following vortexing.

Step 8 was incubation at 65°C for 10 minutes with mixing 2-3 times during the incubation time. Step 9 required the addition of 130 µl of the precipitation AP2 buffer that was increased to 175 µl for *Sclerocactus* DNA extraction, followed by incubation on ice for 5 minutes. The combination of the AP2 buffer and cold temperatures precipitated out the detergent, proteins and polysaccharides. The recommendation for step 10 is to centrifuge the lysate for 5 minutes at 14,000 rpm. An amendment to step 10 was made that included an additional 5 minute spin if a pellet had not formed in the tube. Step 11 of the Qiagen protocol also had slight modifications. The supernatant was carefully pipetted avoiding the pellet and transferred to the lilac QIAshredder mini spin column in a 2 ml collection tube and centrifuged for 2 minutes at 14,000 rpm. The initial spin may result in the column becoming clogged. If the column became clogged, a second spin for 2

minutes at 15,000 rpm was added. If the column remained blocked after the second spin, physical removal of the blockage was attempted using a sterile pipette tip and spinning an additional 2 minutes at 15,000 rpm. If these extra steps did not successfully remove the blockage, the lysate was transferred to a new column and the spinning steps were repeated. Step 12 remained the same, with the flow through lysate from step 11 transferred into a new 2 ml tube. The Qiagen protocol required 1.5 volumes of neutralizing AP3 buffer be added to the lysate in step 13. This volume used for the *Sclerocactus* DNA extraction was 1100 µl AP3, even though the lysate volume may be more than 730 µl. Step 14 involved taking 650 µl of the mixture from step 13 and transferring it to the white DNeasy Mini spin column in a 2 ml collection tube. The column was centrifuged for 1 minute at 8,000 rpm and the flow-though was discarded. An additional spin of 1 minute at 15,000 rpm was added to step 14 to rectify any blockage of the white column. Step 15 repeated step 14 until all the mixture has gone through the spin tube. This step collected the DNA in the column membrane for washing and eluting. The white DNeasy Mini spin column was then transferred to a new 2 ml collection tube in step 16 and 500 µl Buffer AW was added and centrifuged for 1 minute at 8,000 rpm. Step 17 added another 500 µl Buffer AW to the spin column and centrifuges at 14,000 rpm for 2 minutes. Steps 16 and 17 wash the DNA in preparation for elution. Step 18 required the DNeasy Mini spin column be transferred to a 1.5 ml microcentrifuge tube. The Qiagen protocol called for a volume of 100 µl of Buffer AE to be added directly onto the membrane of the column. This was modified slightly to a volume of 75 µl. The column was incubated for 5 minutes at room temperature and then

centrifuged for 1 minute at 8,000 rpm. Step 18 was repeated resulting in a total volume of 150 µl.

The extraction product was visualized on a 1% agarose gel with a 1 KB ladder to verify successful extraction. The procedure was repeated for individuals with unsuccessful extractions where possible. Of the 885 individuals collected for the study, only 16 extractions were unsuccessful even after subsequent extraction attempts.

Microsatellite Marker Design

Microsatellite Library

Genomic DNA was isolated from floral tissue using a modified protocol from the DNeasy Plant Mini Kit (Qiagen). Microsatellite libraries were constructed individually for two taxa, *S. glaucus* and *S. parviflorus.* Isolation of microsatellite loci was performed following the subtractive hybridization method of Hamilton et al. (1999) with some modifications. Digested DNA was enriched for eight oligonucleotide repeats $(AC)_{15}$, $(AG)_{15}$, $(AT)_{15}$, $(CG)_{15}$, $(CCG)_{10}$, $(AAC)_{10}$, $(AGG)_{10}$, and $(CAC)_{10}$. Fragments were cloned using pBluescript II SK- Phagemid vector and the XL1-Blue MRF' bacterial host strain (Agilent Technologies). Color-positive clones were screened for microsatellite regions using a membrane based 'dot blot' method (Glenn and Schable 2002) and the Phototope chemiluminescent detection system (New England Biolabs). A total of 413 positive clones were screened for insert size by PCR using a Master Cycler ProS (Eppendorf). The 20 µl reactions contained 1 µl template DNA, 0.80 µM each of primers T3 and T7 (Integrated DNA Technologies), 1x Thermopol Reaction Buffer (New England Biolabs), 200 µM of each dNTP, and 0.20 units of GoTaq Flexi DNA polymerase (Promega). Clones that exhibited a single amplified band of 400-1000 bp

were cleaned using enzymatic cleanup procedure outlined by Fermentas Molecular Biology (Werle et al. 1994) and sequenced using the T3 primer and BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) in 1/16 volume reactions. Sequences were electrophoresed on a 3730 Genetic Analyzer (Applied Biosystems). For inserts containing a di- or tri-nucleotide microsatellite motif, the T7 primer was used to generate a complementary reverse sequence. All sequences were aligned using SEQUENCHER 5.0 (GeneCodes).

Primer Design

The fragments were analyzed for microsatellites containing 8 or more repeating units. Of the 385 sequenced fragments, only 83 proved suitable for primer design. Primers were designed using PRIMER 3 (Rozen and Skaletsky 2000). One primer of each pair was designed with a common tag at the 5' end following the procedure of Boutin-Ganache et al. (2001; Table 1). Three common tags were used: M13R (AGGAAACAGCTATGACCAT), T7 (GCTAGTTATTGCTCAGCGG), and CAGT (ACAGTCGGGCGTCATCA). We chose ten primers from *S. glaucus* and three primers from *S. parviflorus* that amplified variable microsatellite loci consistently. Loci were amplified with a common tag containing one of three fluorescent dyes, 6-FAM, PET, or VIC (Applied Biosystems).

Table 1. Primer sequences and diversity statistics for 10 micro satellite loci isolated from *Sclerocactus glaucus* (SCGL) and 3 microsatellite loci isolated from *S. parviflorus* (SCPA)

Locus	GenBank Accession Number	Primer sequence (5'-3')	5' Tag	Repeat motif	Allele size range	Species	N_A	H_O	H_E	HWE *P value*	Null Allele
SCGL_71	JX402776	F-TCATCTGGTCCAATCAGCAA	CAGT	$(CT)_{18}$	176-216	SCGL	11	0.66	0.86	0.152	yes
		R- TCAGCGAACAAGAATCATGC				SCPA	7	0.50	0.78	0.018	no
						Mean	**9**	**0.58**	**0.82**		
SCGL_337	JX402777	F- TGAACTTGCTTAGATTTCCCTTA	T7	$(GT)_5TTT(GT)_{10}$	181-239	SCGL	7	0.52	0.70	0.425	no
		R- CGCTAACCCAACACTTTGCT				SCPA	6	0.70	0.74	0.597	no
						Mean	**6.5**	**0.61**	**0.72**		
SCGL_346	JX402778	F- ACTGTGTGGTCGATGAGGAG	CAGT	$(TG)_3TA(TG)_4$	206-244	SCGL	7	0.41	0..65	0.039	yes
		R- AGAAGTGTTGAAGGAGGCAAA				SCPA	4	0.10	0.34	0.001	yes
						Mean	**5.5**	**0.26**	**0..50**		
SCGL_401	JX402779	F- CACAACTTTGCTTCCTGGTTT	CAGT	$(TG)_{27}$	176-258	SCGL	12	0.42	0.73	0.024	yes
		R- CATTTGCATCATATCCACCTAATAAATAAG				SCPA	5	0.70	0.60	0.008	no
						Mean	**8**	**0.56**	**0.66**		
SCGL_416	JX402780	F- CGAACCATCCCCAAAAGTTA	M13R	$(AG)_{11}$	182-208	SCGL	4	0.28	0.67	0.001	yes
		R- GACCCTCTCACCCACAAT				SCPA	6	0.90	0.75	0.890	no
						Mean	**5**	**0.59**	**0.71**		
SCGL_446	JX402781	F- ACTCAAGGTCCATCAAAACA	M13R	$(GA)_{17}$	160-196	SCGL	11	0.45	0.77	0.001	yes
		R- ACTGCCCAATATCGTCTAAA				SCPA	11	0.30	0.90	0.010	yes
						Mean	**11**	**0.38**	**0.84**		
SCGL_448	JX402782	F- GGGTTTCAAGTTCCCCCTTA	T7	$(TGA)_4AGGATTAGGCGTAT(TGA)_3$	282-315	SCGL	3	0.34	0.52	0.159	no
		R- AGTGCCAAGCGAGTTTCATT				SCPA	2	0.00	0.44	0.014	no
						Mean	**2.5**	**0.17**	**0.48**		

Table 1 (continued)

Locus	GenBank Accession Number	Primer sequence (5'-3')	5' Tag	Repeat motif	Allele size range	Species	N_A	H_O	H_E	HWE *P value*	Null Allele
SCGL_450	JX402783	F- TTTTCATGCCCTATGACTATACAA	T7	$(GT)_9$	185-201	SCGL	6	0.72	0.78	0.511	no
		R- GGTTCCACCACCAATTATCC				SCPA	4	0.40	0.34	0.996	no
						Mean	**5**	**0.56**	**0.56**		
SCGL_461	JX402784	F- GGCACTCTATCTCTCTCCCT	T7	$(CT)_{13}$	140-188	SCGL	12	0.55	0.84	0.158	yes
		R- AGGGTTTCATCCACACAAC				SCPA	10	0.60	0.86	0.363	no
						Mean	**11**	**0.58**	**0.85**		
SCGL_704	JX402785	F- GCAAACCATTCAAAGCAGTG	T7	$(CT)_{23}$	199-267	SCGL	15	0.79	0.88	0.191	no
		R- CTTGCTGGCTGTTGAACTA				SCPA	6	0.70	0.71	0.180	no
						Mean	**10**	**0.74**	**0.80**		
SCPA_125	JX402786	F- GGTTCAGCTTGAATAGGTTAATTTC	CAGT	$(CA)_8(GA)_3$	247-297	SCGL	4	0.48	0.57	0.001	no
		R- GGTTGAAACTAGGGGTCAG				SCPA	8	0.78	0.84	0.400	no
						Mean	**6**	**0.63**	**0.70**		
SCPA_268	JX402787	F- GGAGTTCATCAGTAGCCTCT	M13R	$(AG)_3GAGAC$ $(AG)_3AA(GA)_5$	159-185	SCGL	2	0.41	0.37	0.394	no
		R- GGTTGAAACTAGGGGTCAG				SCPA	2	0.30	0.26	0.577	no
						Mean	**2**	**0.36**	**0.32**		
SCPA_268	JX402788	F- CTGTAAGCAGCCGTCGTTG	M13R	$(GA)_3AA(GA)_6(CT_2$ $(GA)_4TA(GA)_8$	209-231	SCGL	3	0.86	0.61	0.030	no
		R- TCTCTCCCCACGCTCTCTTA				SCPA	5	0.60	0.72	0.217	no
						Mean	**4**	**0.73**	**0.66**		

Shown are loci names, the GenBank accession numbers, the forward (F) and reverse (R) primer sequence, the 5' tag used for incorporation of the fluorescent tag M13R (AGGAAACAGCTATGACCAT)), T7 (GCTAGTTATTGCTCAGCGG) or CAGT (ACAGTCGGGCGTCATCA), repeat motif of the sequenced clone, allele size range in base pairs, the number of alleles (N_A), observed heterozygosity (H_O), and expected heterozygosity (H_E) determined as the mean value from 151 total individuals in 7 populations of *S.glaucus* (SCGL;110) and *S. parviflorus* (SCPA: 41, *P value* associated with departure from Hardy-Weinberg Equilibrium (HWE) and the inferred presence of null alleles.

Determining Variability of Microsatellite Loci

One sample population each of *S. glaucus* and *S. parviflorus* were used to evaluate variability in the isolated microsatellite loci. Microsatellite loci were amplified either in 10 µl reactions using the Type-it Microsatellite PCR Kit (Qiagen) or in 12µl reactions using the Fluorescent Tag Microsatellite PCR Protocol (Glenn and Schable, 2005). When possible multiplex PCR with 2-4 loci was used. PCR products were diluted with water and mixed with Hi-Di formamide and LIZ 500 size standard (Applied Biosystems) before electrophoresis on a 3730 Genetic Analyzer. Fragments were sized using PEAK SCANNER v1.0 (Applied Biosystems). We calculated observed (H_O) and expected (H_E) heterozygosity, and tested for deviation from Hardy-Weinberg equilibrium (HWE) using GENALEX v 6.3 (Peakall and Smouse 2006, 2012). Linkage disequilibrium was tested using GENEPOP (Raymond and Rousset 1995; Rousset 2008). MICRO-CHECKER version 2.2.3 was used to infer the presence of null alleles with 1000 bootstrap replicates (Van Oosterhout et al. 2004).

All thirteen microsatellite loci were variable and polymorphic among 151 individuals from 7 selected populations (EC1/2: 43, GJA:15, GR: 30, HH:8, , KE:13, MB:14, UR:28). The mean number of alleles per locus ranged from 2 to 11, with an average of 6.6 (Table 1). The observed and expected mean heterozygosity ranged from 0.00 to 0.90 and 0.26 to 0.90, respectively. There was no evidence of linkage disequilibrium (data not shown). Only one locus, SCGL_446, exhibited a significant deviation from Hardy-Weinberg equilibrium ($p < 0.01$) in both populations. Deviations from HWE were expected due to small isolated populations with limited opportunities for gene flow. Potential null alleles were observed in both species for two loci (SCGL_446,

SCGL_346). Three additional *Sclerocactus* species were amplified using these loci, including *S. brevispinus* and *S. wetlandicus* that were once included in *S. glaucus* and are also listed as threatened (U.S. Department of Agriculture [USDA], 2011). The thirteen markers listed in this paper were used for analysis of *Sclerocactus* genetic diversity, population structure, hybridization, and evolutionary histories.

CHAPTER III

MICROSATELLITE ANALYSIS

Introduction

Habitat destruction due to energy development or urbanization can be devastating to small populations of endangered and threatened species. Anthropogenic activities may lead to habitat fragmentation effectively isolating populations and decreasing the ability to maintain diversity through interbreeding with other populations (Tepedino et al. 2010). Although isolation in various forms is one of the driving forces of speciation, it is of concern when isolation occurs as a result of human interaction (Rhymer and Simberloff 1996). In addition, species that have previously been isolated through natural processes may be brought back into contact through manmade corridors that allow for unnatural gene flow (Rhymer and Simberloff 1996). Describing species based on morphology or location has been found to be unreliable (Sotuyo and Lewis 2007) and therefore land managers need additional information from genetic investigations to answer specific questions about hybridization, gene flow and diversity.

Sclerocactus (Cactaceae) is a genus of 15 species with morphological similarities and overlapping distributions (Heil and Porter 2004; Hochstätter 1993). *Sclerocactus glaucus*, the Colorado hookless cactus, is currently listed as threatened under the U.S. Endangered Species Act (USFWS 1979). *Sclerocactus glaucus* has a relatively small distribution with populations located in Colorado in Montrose, Mesa, Delta and Garfield counties (USDA 2011). Populations of *S. glaucus* are located in areas where resource

exploration, urbanization and cattle grazing are contributing to the loss of habitat. In addition, there are concerns that activity in the area is contributing to possible hybridization with a closely related and widespread species, *Sclerocactus parviflorus* Clover and Jotter.

A genetic investigation of *S. glaucus* is necessary to assess genetic introgression levels with *S. parviflorus,* as well as to determine if taxonomic identification based on morphological characteristics has resulted in species misidentification in selected populations. Understanding the genetic structure within and between these two *Sclerocactus* species will help to direct conservation and land management efforts efficiently. Porter et al.'s (2000) chloroplast trnL-trnF sequence research was used initially to determine the phylogeny of *Sclerocactus*. The study yielded unresolved phylogentic trees, indicating that the chloroplast genome has had minimal genetic changes since divergence of *S. glaucus* and *S. parviflorus* (Porter et al. 2000). However, further genetic investigation may give insights as to if *S. glaucus* and *S. parviflorus* have been separate long enough and have had significant genetic divergence to be considered distinct and separate species. Following the inconclusive resolution of *Sclerocactus* studies, Porter's suggestion was to gain information on genetic structure and how it relates to morphologies in *Sclerocactus* species (Porter et al. 2000*).* Research using *Sclerocactus* morphological characteristics showed that measurements in flower size and spine length were significantly different even though they look similar (Porter et al. 2007). AFLP data confirmed that *S. glaucus* has been isolated from closely related *Sclerocactus* species in Utah (Porter et al. 2007), but has not previously been compared to *S. parviflorus* in Colorado. Porter et al. (2007) recognized that the AFLP data was limited

due to small sample sizes and suggested using sequence analysis over multiple loci for sample sizes of 35 individuals per population.

For this study, some chloroplast sequence analysis was done (see Chapter IV) along with the analysis of 13 polymorphic microsatellite markers to examine population genetic structure within and among *S. glaucus* and *S. parviflorus* populations (Schwabe et al. 2012). Other genetic information was assessed such as genetic diversity, gene flow, and hybridization between *S. glaucus* and *S. parviflorus*. The markers were used across 865 individuals from 38 distinct populations and based on the data collected suggestions will be made as to which populations should have conservation priority. The goal of this study is to assess populations of *S. glaucus* in order to give conservation managers recommendations on which populations to target in order to maintain and preserve the species.

Methods

Population Sampling

Staff from Denver Botanic Gardens, the U.S. Fish and Wildlife Service and the Bureau of Land Management collected samples from 38 populations of *Sclerocactus* including *S. glaucus, S. parviflorus* and *S. cloveriae* (*S. cloveriae* collected by Ken Heil). Floral tissue was taken from plants with more than one flower or bud. Tissue was stored in plastic bags containing silica gel with the plant population name and identification number on the bag. A photographic record and GPS coordinates were taken for each individual collected. The goal was to sample 30 individuals per population or the maximum number of individuals with two or more floral buds. However, some of the populations did not contain enough individuals or the individuals present did not have

more than 2 flower buds. Figure 1 shows the population locations and Table 2 lists the number of individuals sampled from 28 populations of *S. glaucus*, 9 populations of *S. parviflorus*, and 1 population of *S. cloveriae*, which is considered by some as a New Mexico variety of *S. parviflorus* (USDA 2011).

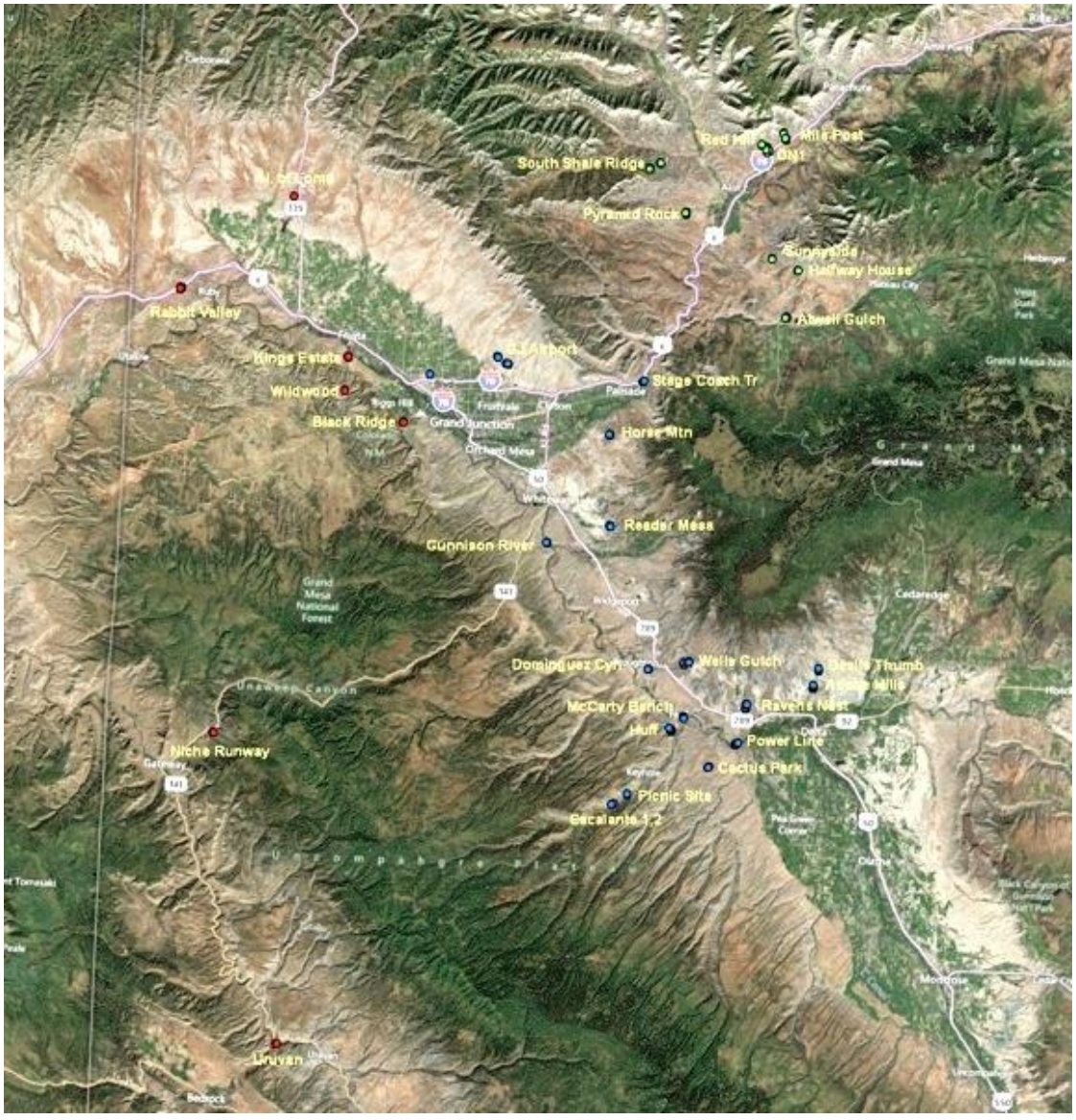


Figure 1: Population distribution map for 35 populations of *SCGL* and *SCPA* located in Colorado. Colors correspond to genetic clusters that have been resolved by STRUCTURE analysis (Figure 3). Populations located outside Colorado are not shown (La Sal, Shiprock and Farmington).

Table 2. The populations used in this study with the species name, population name, abbreviation, state, and county where the population is located, and numbers of individuals used in these populations

Species		Location	County	N
S. glaucus				
GLDT	Devils Thumb	Colorado (S)	Delta	18
GLAH	Adobe Hills	Colorado (S)	Delta	30
GLRN	Ravens Nest	Colorado (S)	Delta	30
GLPL	Powerline	Colorado (S)	Delta	29
GLCP	Cactus Park	Colorado (S)	Delta	30
GLEC1	Escalante Cyn 1	Colorado (S)	Montrose	13
GLEC2	Escalante Cyn 2	Colorado (S)	Montrose	30
GLPS	Picnic Site	Colorado (S)	Delta	30
GLHU	Huff	Colorado (S)	Delta	27
GLMB	McCarty Bench	Colorado (S)	Delta	14
GLWG	Wells Gulch	Colorado (S)	Delta	30
GLDC	Dominguez Cyn	Colorado (S)	Delta	30
GLGR	Gunnison River	Colorado (S)	Mesa	30
GLWW	Reeder Mesa	Colorado (S)	Mesa	29
GLHM	Horse Mountain	Colorado (S)	Mesa	30
GLGJA	GJ Airport	Colorado (S)	Mesa	15
GLSCT	Stage Coach Trail	Colorado (N)	Mesa	27
GLAG	Atwell Gulch	Colorado (N)	Mesa	30
GLHH	Halfway House	Colorado (N)	Mesa	8
GLSUN	Sunnyside	Colorado (N)	Mesa	4
GLPR	Pyramid	Colorado (N)	Mesa	30
GLSRP	S. Shale Ridge Pond	Colorado (N)	Mesa	30
GLSSR	S. Shale Ridge	Colorado (N)	Mesa	24
GLSTJ	S. Shale Ridge T-Junction	Colorado (N)	Mesa	23
GLRH	Red Hill	Colorado (N)	Garfield	26
GLON1	ONIE/R	Colorado (N)	Garfield	28
GLMP	Milepost 68	Colorado (N)	Garfield	19
S. parviflorus				
PAWT	Black Ridge	Colorado	Mesa	29
PALCT	Wildwood	Colorado	Mesa	11
PAKE	Kings Estate	Colorado	Mesa	13
PANL	North of Loma	Colorado	Mesa	10
PARV	Rabbit Valley	Colorado	Mesa	29
PANR	Niche Runway	Colorado	Mesa	11
PAUR	Uruvan	Colorado	Montrose	28
PALS	La Sal	Utah	San Juan	30
PALS	Shiprock	New Mexico	San Juan	20
S. cloveriae				
CLFA	Farmington	New Mexico	San Juan	20

DNA Analysis

DNA was successfully extracted from 865 individuals from 38 populations using a modified version of the DNeasy Plant Mini DNA extraction kit (Qiagen, Valencia CA; see Chapter II).

Microsatellite PCR

A microsatellite library was designed and primers were developed to amplify 13 variable loci in *Sclerocactus* (Schwabe et al. 2012). Details of the development procedures performed are detailed in Chapter II. The primer pairs were optimized for annealing temperatures and either magnesium concentrations ($MgCl_2$ or $MgSO_4$) or Qiagen Multiplex PCR kit (Qiagen, Valencia CA). PCR amplifications were carried out with 12 µL reactions using magnesium or 10 µL reactions using the Qiagen kit. The magnesium reactions included 1 µL genomic DNA, 0.60 µL non-tagged primer (5µM)**,** 0.60 µL tagged primer (0.50 µM)**,** 0.70 µL dNTP mixture (at 2.5 mM)**,** 0.06 µL Taq polymerase (Promega, Madison, WI, USA), 2.4 µL GoTaq Flexi Buffer (Promega, Madison, WI, USA)**,** 4.98-5.48 µL dH_2O, 1 µL MgCl or 0.50 µL of $MgSO_4$, 0.60 µL fluorescent tag (5µM; M13 or CAGT tag, with a 6-FAM or VIC label) and 0.06 µL BSA (Bovine Serum Albumin, 100X)**.** The Qiagen Multiplex PCR kit reactions included 1 µL genomic DNA, 0.25 µL fluorescent tag (5µM; M13 or CAGT tag, with a 6-FAM or VIC label), 0.50 µL 20X primer, 5.0 µL of Qiagen Multiplex PCR Master Mix (Qiagen , Valencia CA), 0 µL or 1 µL Q-solution (Qiagen , Valencia CA) and 2.25-3.25 µL dH_2O. Optimized amplification temperatures and magnesium concentrations ranges are shown in Table 3.

Table 3. Conditions for 13 primer pairs to amplify 13 variable . microsatellite markers for *Sclerocactus glaucus* and *S. parviflorus* individuals

Primer	Tag	Label	DNA	MgCl/MgSO2	Anneal
SCGL-71	CAGT	FAM	1µl	1 µl MgCl	52.9
SCGL-125	CAGT	VIC	1µl	QIAGEN -Q	mid
SCGL-337	T7	PET	1µl	QIAGEN-no Q	mid
SCGL-346	CAGT	FAM	1µl	2µl MgCl	59.6
SCGL-401	CAGT	PET	1µl	QIAGEN-Q	mid
SCGL-416	M13	VIC	1µl	4 µl MgCl	59.6
SCGL-446	M13	PET	1µl	2 µl MgCl	57.4
SCGL-448	T7	VIC	1µl	2µl MgCl	57.4
SCGL-450	T7	VIC	1µl	2 µl MgCl	57.4
SCGL-461	T7	PET	1µl	QIAGEN-Q	mid
SCPA-268	M13	FAM	1µl	4 µl MgCl	55.1
SCPA-623	M13	FAM	1µl	2µl MgCl	50.9

A PCR master mix was prepared with enough reagents for all reactions in a 96 well plate, with one well reserved as a negative control**.** PCR amplification was carried out on an Eppendorf Mastercycler proS (Hamberg, Germany). An initial 5 minute denaturing step was followed by thirty five amplification cycles with a 1 min denaturing at 95º C, 1 min annealing at primer-specific temperatures and 1 min extension at 72ºC. Amplification products were verified visually on a 1% agarose gel. Fluorescently labeled PCR products were multiplexed where possible and analyzed on an Applied Biosystems 3130 Genetic Analyzer at Arizona State University. Products were loaded along with GeneScan 500LIZ Size Standard (Applied Biosystems, Foster City, CA) according to manufacturer's specifications. PeakScanner ver. 1.0 (Applied Biosystems, Foster City, CA) was used to score the size of each fragment. The size of each allele was recorded using a Microsoft Xcel spreadsheet.

Statistical Analysis

Linkage disequilibrium was tested using GENEPOP ver. 4.0.10 (Raymond and Rousset 1995; Rousset 2008). GENALEX ver. 6.3 (Peakall and Smouse 2006, 2012) was used to calculate deviation from Hardy-Weinberg equilibrium (HWE), average number of alleles (A), effective number of alleles (A_e), observed heterozygosity (H_O), expected heterozygosity (H_e), inbreeding coefficient (F_{IS}) and pairwise genetic distance between populations (F_{ST}).

GENALEX ver. 6.3 (Peakall and Smouse 2006, 2012) was also used to generate a principle component analysis (PCoA). Principle component analysis is a multivariate analysis used to investigate genetic diversity using markers such as microsatellites (Jombart and Dufour 2009). It uses biological processes such as genetic diversity and assigns a spatial genetic structure using a data matrix. The data matrix is created using data from the variable microsatellite markers for each individual or population in the data set. The results are graphed and can then be used to make inferences about genetic patterns of diversity and population structuring (Jombart and Dufour 2009).

Population structure was analyzed using the Bayesian cluster analysis software program STRUCTURE ver. 2.3.2 (Pritchard et. al 2000). Burn-in and run lengths of 50,000 replicates were used for each STRUCTURE analysis. The number of inferred populations (*K*) was determined using STRUCTURE HARVESTER (Earl and vonHoldt 2012). STRUCTURE HARVESTER is a web-based program designed to visualize *K* values from multiple iterations using the Evanno et al. (2005) method. This method uses an algorithm that compares the rate of change of log-likelihood values between successive *K* values over consecutive iterations. This allows a K value to be assigned

based on the greatest rate of change and the graphs generated indicate which number of genetic groups (*K*) best fit the data (Earl and vonHoldt 2012).

Bar graphs generated in STRUCTURE indicate genetic information using colors. The number of colors is equal to the K value. Each individual is represented as a color that corresponds to the genetic information gathered from the microsatellite data. Each population is labeled and a thin vertical line represents each individual. Individuals with multiple colors indicate genetic signals from multiple groups. A hybrid individual will have a significant signal from at least 2 groups. Small amounts of signal from both species are to be expected due to the relatively recent divergence of the two species. The unresolved phylogenetic tree that Porter et al. (2000) generated from chloroplast data, it is assumed that this genus is recently divergent. Therefore an expectation of more than 25% signal from *S. parviflorus* will be used to define a hybrid individual. However, for the analyses ranges of 10-25% and 26-50% will be used to infer minimal hybridization and substantial hybridization, respectively.

Results

DNA extraction was 98.2% successful with only 16 individuals out of 883 collected specimens showing no DNA bands visible from 2 µL run on a 1% agarose gel. After re-extraction attempts were made, the remaining 16 unsuccessful extractions ranged from 1-3 individuals in Devils Thumb, Powerline, Escalante Canyon 1, Huff, Reeder Mesa, Sunnyside, Red Hill, Milepost 68, North of Loma, Rabbit Valley and Uruvan populations. The single *S. glaucus* and *S. parviflorus* specimens collected from the Denver Botanic Gardens were not used in final analyses because the origin was unknown. All thirteen microsatellite loci were variable and polymorphic among populations.

Evidence of linkage disequilibrium was minimal with 39 out of 2886 comparisons showing signal of linkage ($p < 0.01$). Of the 39 comparisons with linkage disequilibrium, 14 of these were located in the La Sal *S. parviflorus* population. Locus by population comparisons revealed that 221 out of 481 total comparisons were outside HWE ($p < 0.01$). Deviation from HWE is expected to some extent due to inbreeding, small population sizes and overlapping generations. However, two loci (446 and 623) have excessive deviation (35 and 21 out of 38 populations respectively) indicating there may be inconsistent mutation patterns and/or scoring errors for these loci. The following research analyses give support to the division of microsatellite data into three groups. The three distinct data groups are referred to as *S. parviflorus,* north *S. glaucus* and south *S. glaucus.* The *S. parviflorus* group contains *S. cloveriae*, and the north and south *S. glaucus* groups are divided according to the supporting data below.

Diversity

Table 4 contains calculations for each population for average number of alleles (A), effective number of alleles (A_e), observed heterozygosity (H_o), expected heterozygosity (H_e) and inbreeding coefficient (F_{IS}). The average number of alleles across all *S. glaucus* and *S. parviflorus* populations was 7.21 and 6.3, respectively (Table 4). Among *S. glaucus,* Domingez Canyon had the highest number of alleles (9.15) and Sunnyside had the lowest (2.54). The average effective number of alleles across all *S. glaucus* and *S. parviflorus* populations was 4.26 and 3.57, respectively (Table 4). Among *S. glaucus,* Powerline had the highest number of effective alleles (5.54) and Sunnyside had the lowest number of effective alleles (2.19).

Table 4. Genetic diversity statistics generated from GENALEX ver. 6.3 (Peakall and Smouse 2006, 2012) from all sampled populations for 13 microsatellite loci

Species	Location	N	A	A_e	H_o	H_e	F_{is}
S. glaucus							
Devils Thumb	Colorado (S)	18	6.69	4.59	0.47	0.68	0.31
Adobe Hills	Colorado (S)	30	8.38	5.16	0.44	0.68	0.35
Ravens Nest	Colorado (S)	30	8.62	5.01	0.58	0.70	0.18
Powerline	Colorado (S)	29	9.08	5.54	0.49	0.72	0.31
Cactus Park	Colorado (S)	30	8.15	4.84	0.50	0.69	0.27
Escalante Cyn 1	Colorado (S)	13	5.46	3.82	0.48	0.66	0.28
Escalante Cyn 2	Colorado (S)	30	7.46	3.97	0.53	0.69	0.24
Picnic Site	Colorado (S)	30	7.46	3.85	0.50	0.68	0.26
Huff	Colorado (S)	27	8.08	4.76	0.53	0.69	0.23
McCarty Bench	Colorado (S)	14	6.92	4.59	0.53	0.66	0.20
Wells Gulch	Colorado (S)	30	8.54	5.02	0.53	0.70	0.24
Dominguez Cyn	Colorado (S)	30	9.15	4.96	0.50	0.70	0.28
Gunnison River	Colorado (S)	30	7.92	4.36	0.50	0.64	0.22
Reeder Mesa	Colorado (S)	29	9.08	5.27	0.42	0.69	0.40
Horse Mountain	Colorado (S)	30	7.38	4.52	0.36	0.67	0.46
GJ Airport*	Colorado (S)	15	7.00	4.15	0.48	0.60	0.20
Stage Coach Trail*	Colorado (S)	27	7.23	4.38	0.41	0.68	0.39
Atwell Gulch	Colorado (N)	30	7.46	3.54	0.42	0.65	0.35
Halfway House	Colorado (N)	8	3.77	2.72	0.51	0.57	0.11
Sunnyside	Colorado (N)	4	2.54	2.19	0.40	0.46	0.14
Pyramid	Colorado (N)	30	6.85	3.81	0.47	0.62	0.24
S. Shale Ridge Pond	Colorado (N)	30	6.85	3.66	0.40	0.65	0.39
S. Shale Ridge	Colorado (N)	24	7.85	4.14	0.45	0.69	0.35
S. Shale Ridge T-Junction	Colorado (N)	23	7.15	4.29	0.43	0.65	0.34
Red Hill	Colorado (N)	26	6.69	3.87	0.46	0.61	0.25
ONIE/R	Colorado (N)	28	7.62	4.53	0.49	0.68	0.28
Milepost 68	Colorado (N)	19	5.23	3.56	0.39	0.62	0.38
Mean		**25**	**7.21**	**4.26**	**0.47**	**0.66**	**0.28**
S. parviflorus							
Black Ridge	Colorado	29	7.62	3.60	0.36	0.65	0.45
Wildwood	Colorado	11	4.38	2.74	0.30	0.57	0.48
Kings Estate	Colorado	13	5.46	3.95	0.45	0.65	0.30
North of Loma	Colorado	10	5.85	3.95	0.51	0.64	0.21
Rabbit Valley	Colorado	29	7.23	4.04	0.42	0.66	0.36
Niche Runway	Colorado	11	4.54	2.96	0.32	0.57	0.45
Uruvan	Colorado	28	6.77	3.37	0.34	0.58	0.41
La Sal	Utah	30	7.77	3.90	0.41	0.66	0.38
Shiprock	New Mexico	20	6.15	3.65	0.31	0.60	0.48
Mean		**20**	**6.20**	**3.57**	**0.38**	**0.62**	**0.37**

Table 4 (continued)

Species	Location	N	A	A_e	H_o	H_e	F_{is}
S. cloveriae							
Farmington	New Mexico	20	5.92	3.45	0.35	0.55	0.37

Number of individuals in each population (N) was used to calculate average values for number of alleles (A), effective alleles (A_e), observed heterozygotes (H_o), expected heterozygotes (H_e) and inbreeding coefficients (F_{IS}).
*Grand Junction Airport and Stage Coach Trail were previously identified as *S. parviflorus* populations

The average observed heterozygosity across all *S. glaucus* and *S. parviflorus* populations was 0.47 and 0.38, respectively (Table 4). Among *S. glaucus,* Ravens Nest had the highest observed heterozygosity (0.58) and Horse Mountain had the lowest observed heteozygosity (0.36). The average expected heterozygosity across all *S. glaucus* and *S. parviflorus* populations were 0.66 and 0.62, respectively. Among *S. glaucus,* Powerline had the highest expected heterozygosity (0.72) and Sunnyside had the lowest expected heteozygosity (0.46). The average inbreeding coefficient across all *S. glaucus* and *S. parviflorus* populations was 0.28 and 0.37, respectively (Table 4). The lowest F_{IS} was in Halfway House *(S. glaucus)* population (0.11) and the highest F_{IS} was in the Wildwood and Shiprock populations *(S. parviflorus)* (0.48).

Pairwise genetic distances (F_{ST}) were calculated between all pairs of populations (Table 5). According to Wright (1978) a value of < 0.05 indicates very little genetic differentiation, 0.05-0.15 indicates a small amount of genetic differentiation, 0.16-0.30 indicates populations are moderately differentiated, and > 0.30 indicates populations that are highly differentiated from one another. Research has shown that a group partition of F_{ST} greater than 0.15 is a well supported guideline for separation of species; values lower than this do not distinguish species, but merely subpopulations (Long and Kittles 2003). Hamrick and Godt (1996) compared genetic diversity within and among populations and

found pairwise comparisons among populations were higher, however, their results used allozyme data to compare G_{ST}. Additionally, Sites and Marshall (2004) determined operational criteria for delimiting species including using statistics to partition species. The unit of measure for species boundaries was Nei's genetic distance over multilocus allozyme data (*D* of Nei 1970, 1972) suggesting that genetic distance corresponds to reproductive isolation specifically when groups differ by a value of $D \geq 0.15$ (Highton 2000). Highton recognized this number as arbitrary but that 97% of genetic studies fell within this measure of species delimitation (Sites and Marshall 2004). Although the data in this study is comparing multilocus microsatellite data among populations and species using F_{ST}, there is enough supporting data to suggest that an $F_{ST} \geq 0.15$ is a relatively true measure for determining species boundaries for *Sclerocactus* and many other species. An F_{ST} value of 0.10-0.15 indicates a range generally considered to be members of the same species. All sampled populations were compared to each other and the average genetic distance (F_{ST}) was 0.15. Between all 28 *S. glaucus* populations the average F_{ST} was 0.09, with values of 0.02 and 0.06 between only north and only south *S. glaucus* populations, respectively (Table 5). STRUCTURE and PCoA results encouraged an analysis between the north and south populations to determine the level of differentiation between these two apparently distinct groups. The average F_{ST} value between the all of the north populations and all of the south *S. glaucus* populations was 0.11. The average F_{ST} value between the 9 *S. parviflorus* populations was 0.12 and when comparing *S. glaucus* to *S. cloveriae* the average F_{ST} was 0.19 and comparing *S. parviflorus* to *S. cloveriae* the average F_{ST} was 0.17.

Table 5. Relative measurements of genetic distance (F_{ST}) between sampled groups

Groups	Average F_{ST}
S. glaucus	0.09
S. glaucus North	0.02
S. glaucus South	0.06
S. glaucus North/ South	0.11
S. glaucus/ S. parviflorus	0.15
S. glaucus/ S. cloveriae	0.19
S. parviflorus	0.12
S. parviflorus/ S. cloveriae	0.17

Genetic Structure

Bayesian cluster analyses using STRUCTURE were run for all individuals using K=1 to K=30 to determine the most likely number of genetic clusters. Using STRUCTURE HARVESTER it was determined that K=3 is the most probable assignment for the data set including all individuals (Figure 2a). The complete STRUCURE analysis of all 38 populations divided the genetic data into three clusters (Figure 3). From this analysis the populations were divide into distinct clusters of *S. parviflorus*, including *S. cloveriae* (red), and *S. glaucus* divided into north (green) and south populations (blue). The populations of *S. glaucus,* which are green, are found in the Colorado River drainage near De Beque and the populations of *S. glaucus*, which are blue, are located in the eastern Grand Valley and Gunnison River drainage.

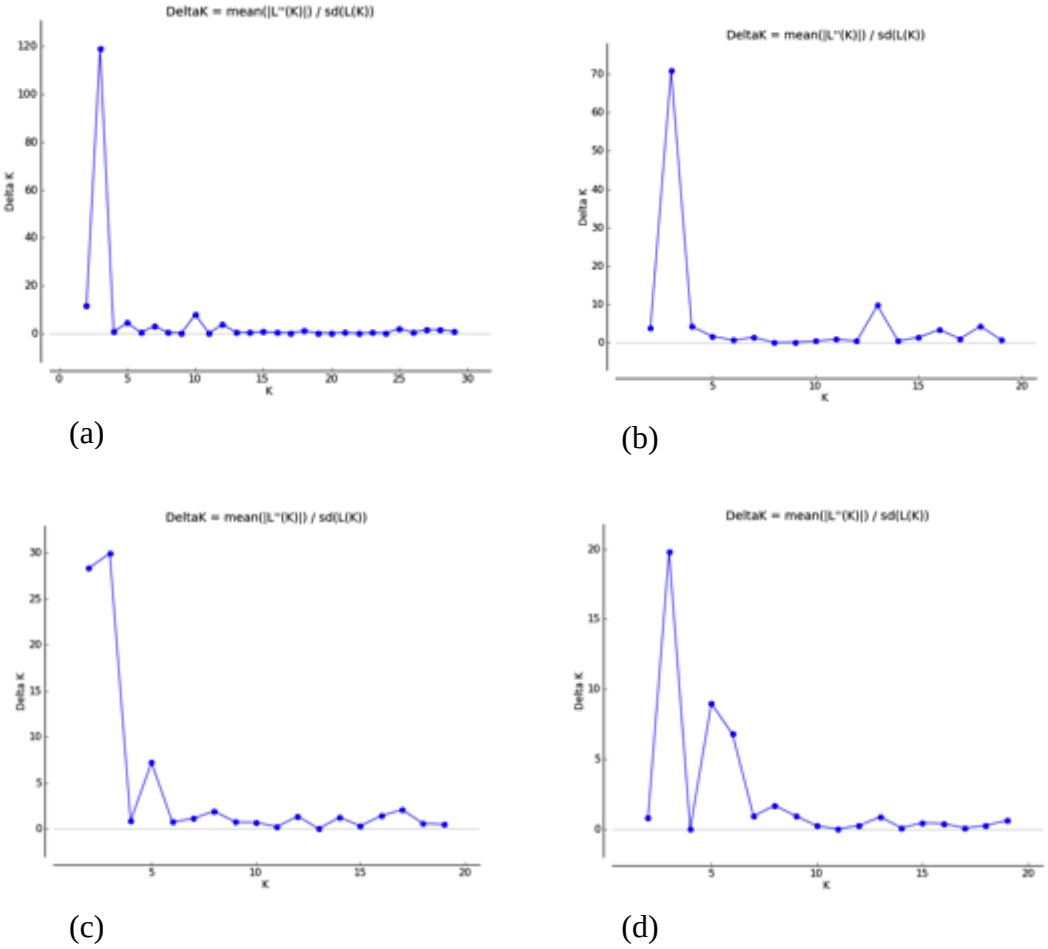


Figure 2: STRUCTURE HARVESTER data indicating the rate of change likelihood calculated using the Evanno et al. (2005) method for each K value assigned, (a) all 38 populations (K=1-K=30), (b) *S. cloveriae* and *S. parviflorus* populations (K=1-K=20), (c) north *S. glaucus* populations (K=1-K=20), (d) south *S. glaucus* populations (K=1-K=20).

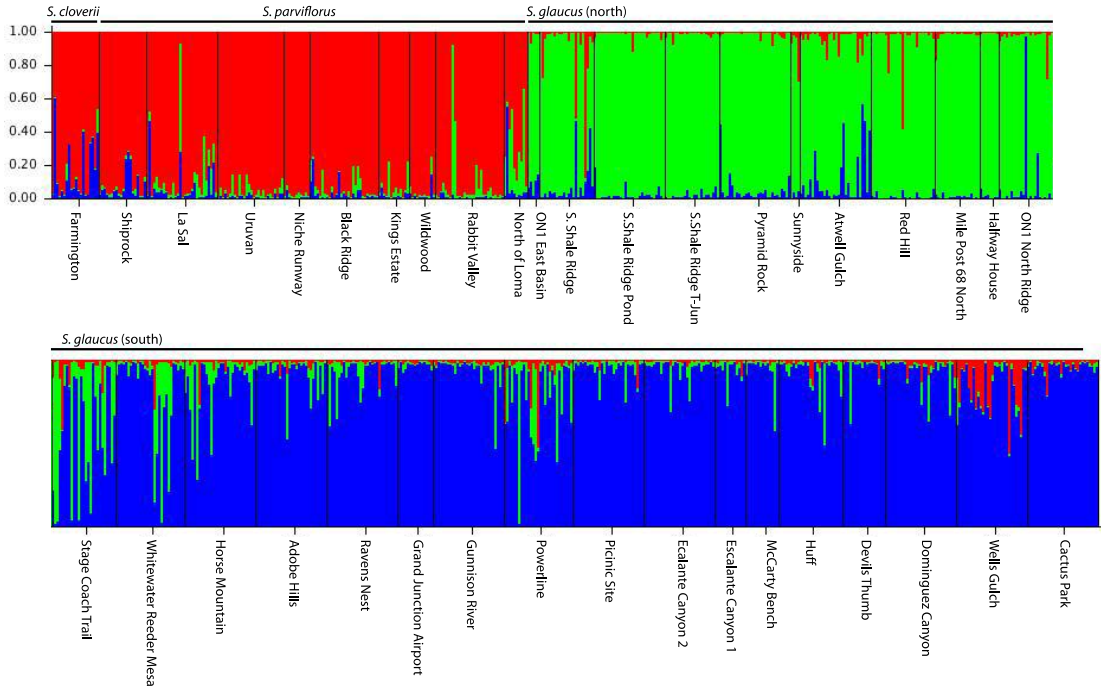


Figure 3: Bar plot images from STRUCTURE analysis indicating inferred population assignment of 865 individuals assigned to K=3 groups for 38 populations.

The data was then broken into smaller subsets of populations to determine additional population structure between the three initial clusters. Using STRUCTURE HARVESTER it was determined that K=3 is the most probable assignment for each of the smaller data sets (Figure 2 b, c and d). These additional analyses were between north *S. glaucus* (Figure 4, K=3), south *S. glaucus* (Figure 5, K=3), and *S. parviflorus* including *S. cloveriae* (Figure 6, K=3). The north *S. glaucus* populations form three groups that are generally located in the west (blue), north (green) and east (red) (Figure 4). The south *S. glaucus* populations also resolve three groups, with less distinction between them, which could be considered loosely as north (red), west (green) and east (blue) (Figure 5). The final STRUCTURE analysis was for *S. parviflorus*, which included *S. cloveriae;* three

clear genetic structure groups form, corresponding to New Mexico (red), Utah (blue) and Colorado (green) populations (Figure 6).

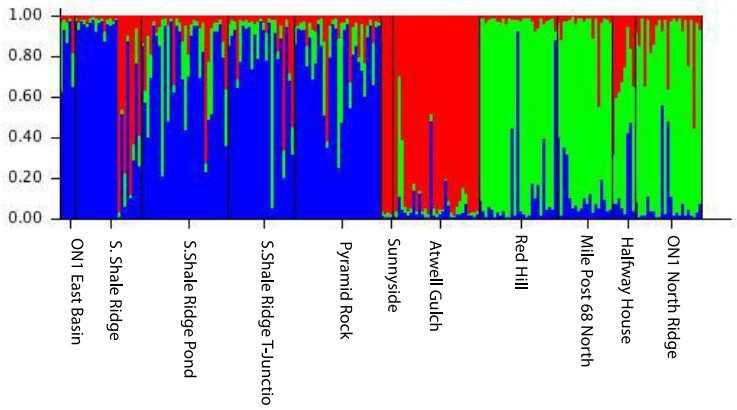


Figure 4: STRUCTURE analysis of 222 individuals from 11 populations from the north *Sclerocactus glaucus* populations assigned to K=3 groups, which are clustered into a west group (blue), a north group (green) and a east group (red).

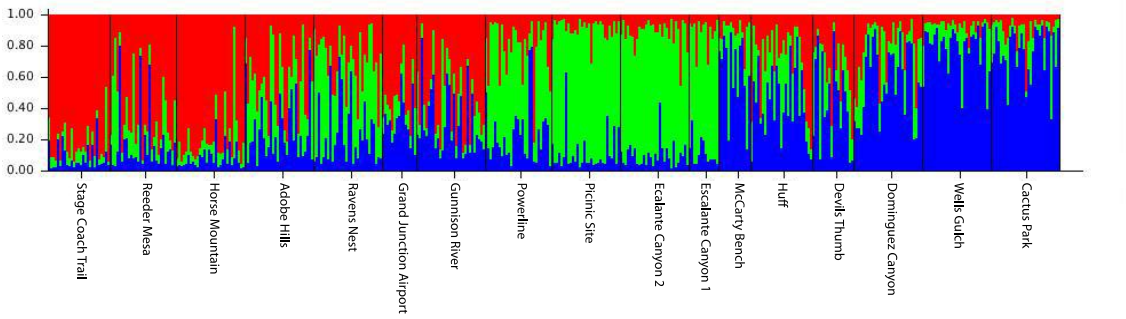


Figure 5: STRUCTURE analysis of 442 individuals from 17 populations from the south *S. glaucus* populations assigned to K=3 groups, which trend toward a north group (red), a west group (green) and a east group (blue).

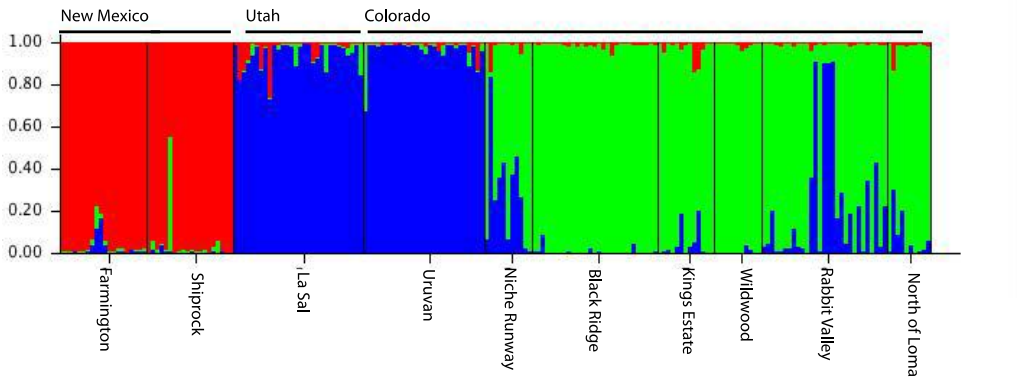


Figure 6: STRUCTURE analysis of 201 individuals from the one *S. cloveriae* and nine *S. parviflorus* populations assigned to K=3 groups, which cluster into a New Mexico group (red), a Utah group (including the far western Colorado population from Uruvan) (blue) and a Colorado group (green).

Hybrid individuals have signal from both *S. parviflorus* (red) and *S. glaucus* (blue and/or green) in the analyses of all populations (Figure 3). The STRUCTURE analysis indicates that some *S. glaucus* populations have introgression from *S. parviflorus*. Levels of hybridization need to be defined, as many *S. glaucus* have a small genetic signal associated with *S. parviflorus*. Of the 664 individuals sampled from *S. glaucus* populations, 27 individuals have 10-25% *S. parviflorus* genetic signal (4%) and an additional 21 individuals have more than 25% *S. parviflorus* genetic signal (3%). The population with the greatest *S. parviflorus* introgression is Wells Gulch with 16 of the 30 individuals having more than 10% *S. parviflorus* signal. It is interesting to note that gene flow is occurring from *S. glaucus* to *S. parviflorus* also. Of the 201 *S. parviflorus* individuals sampled, 25 (12%) have higher than a 25% genetic signal from *S. glaucus.*

Initial concern to conservation biologists was that the overlapping ranges of *S. glaucus* and *S. parviflorus* could result in hybridization. The fear was that if *S. parviflorus* is more abundant and widespread, and if hybridization is occurring, then it

would be possible for *S. glaucus* to disappear due to swamping of genetic material from *S. parviflorus.* Gene flow from *S. glaucus* into *S. parviflorus* populations indicates that the hybridization seen in *S. glaucus* is not necessarily due to the abundance of *S. parviflorus* as previously feared. Moreover, the *S. parviflorus* population, North of Loma, appears to have substantial north *S. glaucus* introgression with every sampled individual having more that 25% *S. glaucus* genetic signal.

The PCoA for the 38 populations resolves three clusters (Figure 7), which correspond to the STRUCTURE analysis (Figure 3). The variation represented by Coord. 1 (x), Coord. 2 (y) and Coord 3 (z) (not shown) are 37.23%, 28.84% and 11.1%, respectively. The *S. parviflorus/S. cloveriae* populations are outlined in red, the south Gunnison River and eastern Grand Valley drainages *S. glaucus* populations outlined in blue, and north *S. glaucus* populations located in the Colorado River are outlined in green. Interestingly, the points on the coordinates mimic the geographical location of the populations. The New Mexican and Utah populations are located toward the edge of the red cluster while the populations that are closer to Grand Junction cluster toward the center of the group. Two distinct clusters from both STRUCTURE and PCoA of the north and south *S. glaucus* populations give further support that even though they are the same species, they are distinct from one another.

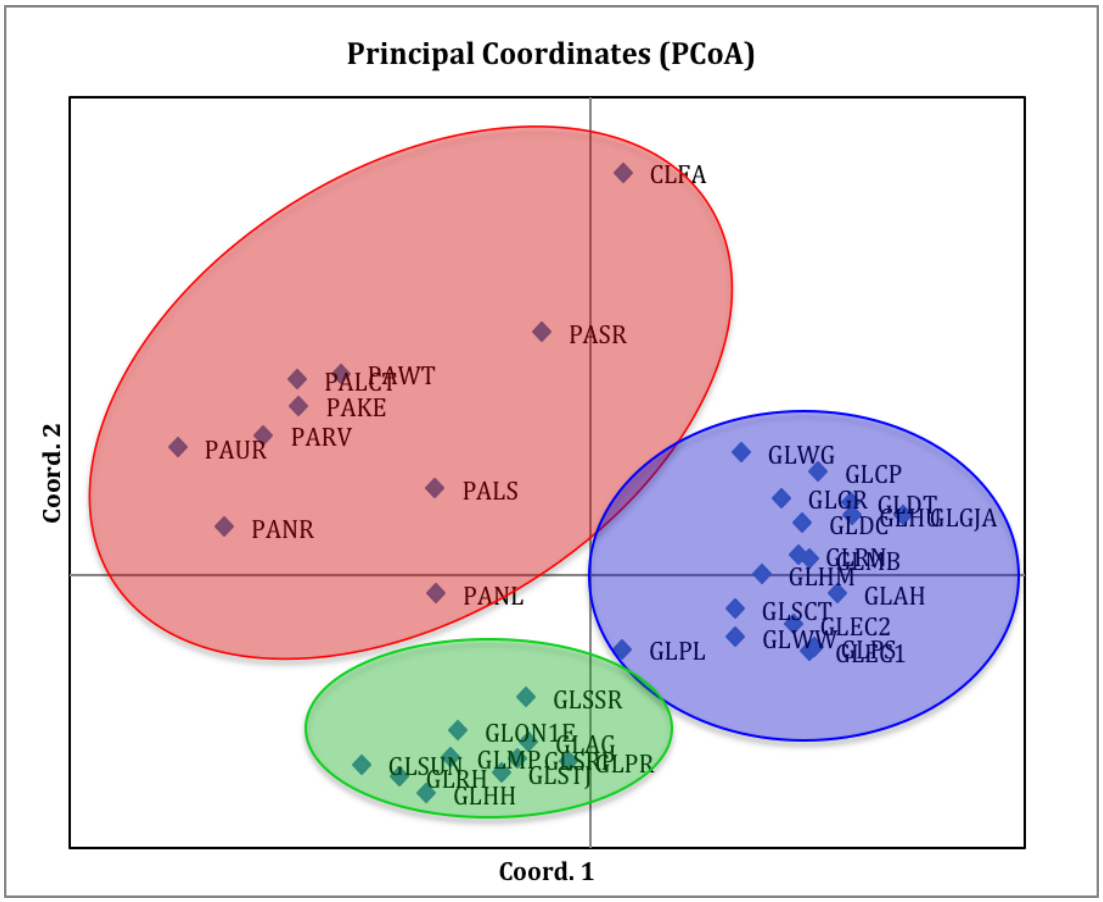


Figure 7: Principle Component Analysis (PCoA) showing spatial genetic structures created from a data matrix of 37 populations and 13 polymorphic microsatellite loci with variation shown on x (37.23%) and y coordinates (28.84%). Populations GLON1E and GLON1R are treated as one population in this analysis.

Discussion

Threatened and endangered species are particularly sensitive to anthropogenic and biological processes that could result in declining numbers. Ecological stability relies on the interaction between multiple species and the loss of any one species within a system may result in significant changes to the system (Tepedino et al. 2010). Hybridization has been reported to contribute to species collapse (Taylor et al. 2006) when once isolated

species come back into contact. Anthropogenic activities such as urbanization, industrial development and resource exploration may open up new corridors for gene flow between previously isolated species (Rhymer and Simberloff 1996; Tepedino et al. 2010). The threatened Colorado hookless cactus, *Sclerocactus glaucus* is experiencing not only habitat loss from resource exploration, cattle grazing and human activities, but may also be hybridizing with a closely related congener due to habitat modification. Management of *S. glaucus* habitat may be necessary for preservation of the species and maintenance of the diversity within and among populations. Management efforts may vary widely from relocation of populations, removing individuals from populations or redirecting development so as not to disturb natural populations. If there is significant hybridization due to the dispersal of seed or entire plants into interspecific populations, a more aggressive strategy may be necessary. However, if the dispersal method leading to hybridization appears to be from pollen movement, then some further investigations into the pollinators and possible man made corridors may need to be addressed. It may be that these two species are naturally hybridizing via pollinators. If this is the case, natural populations can be left alone and managed for preservation of the natural population rather than aggressive conservation measures.

Genetic Structure

Evolutionary groups for the 38 populations were inferred through the use of STRUCTURE. This analysis clearly broke the populations into three distinct genetic clusters that contained 11 north *S. glaucus* populations, 17 south *S. glaucus* populations, and 9 *S. parviflorus* populations grouped with the one *S. cloveriae* population (Figure 3). Principle component analysis reinforced the STRUCTURE results and produced three

distinct genetic groups in alignment with the groups determined by STRUCTURE (Figure 7). These two tests together present robust support for *S. parviflorus* and *S. glaucus* being distinct and separate species and also that the north and south *S. glaucus* groups are unique and distinct from each other.

Processes, which influence genetic distribution within and among any set of samples, can be analyzed using genetic distance (F_{ST}) (Holsinger and Weir 2009). Genetic distance (F_{ST}) has been found to be most useful when the samples, in this case populations of *S. glaucus, S. parviflorus* and *S.* cloveriae, are discrete units as opposed to arbitrary divisions along a continuous distribution (Holsinger and Weir 2009). Additionally, F_{ST} and numbers relating to F_{ST} can be used to establish "the relationship between the recent evolutionary history of populations and environmental or demographic variables" (Foll et al. 2008. Genetic distance (F_{ST}) results support the evolutionary pattern of three distinct groups within these two taxa. Genetic differentiation from genetic analyses have shown that a measure such as $F_{ST} \geq 0.15$ as a criteria for species delimitation is valid (Highton 2000; Long and Kittles 2003; Sites and Marshall 2004). From the data outlined in Table 5 it is clear that *S. glaucus, S. parviflorus* and *S. cloveriae* are all distinct and separate species based on average F_{ST} values (all $F_{ST} >$ 0.15). Moreover, when *S. glaucus* is compared across all populations it has a F_{ST} value of 0.09, indicating some divergence within *S. glaucus*. The populations within the north *S. glaucus* populations (0.02) have almost no differentiation and the south *S.glaucus* populations (0.06) have very limited genetic distance between them. However, when the north and south populations are compared to one another they have much more genetic distance (0.11). The F_{ST} value comparing the north and south *S. glaucus* populations

indicates that these areas hold two distinct genetic clusters of this species. The F_{ST} value between the *S. parviflorus* group (0.12) might be explained due to the very large geographical distance between the populations in this study.

STRUCTURE resolved three distinct evolutionary units that were supported by the PCoA and genetic distance data. Within the three groups, north *S. glaucus*, south *S. glaucus* and *S. parviflorus,* STRUCTURE further separated out additional genetic distinctiveness. These groups appear to have a strong correlation to geographical areas. *Sclerocactus parviflorus* forms clusters of New Mexico, Utah (includes Uravan) and Colorado types, which indicates that geographic differentiation is occurring. These differences may be due to selection pressures in different locations such as variations in habitat, climate or differences in pollinators (Coyne and Orr 2004), or from genetic drift due to isolation by distance. Similar patterns are seen in the north and south *S. glaucus* groups. Within each of these groups there are three genetic clusters that appear to be related to location, forming east, north and west genetic clusters. Since large distances do not separate these populations, the differences in genetic signals are not likely due to differences in climate. The differences could be due to isolating barriers that are not obvious from the map. Another possibility is that populations have increased gene flow within these smaller groups because they are isolated by distance. For example, South Shale Ridge, South Shale T- Junction, South Shale Ridge Pond and Pyramid Rock are all located in the same area and it is intuitive that these populations would have a higher chance of genetic exchange when compared to the populations that are further away. There are also three groups in the south *S. glaucus* populations which are less distinctive. However these groups also appear to be separating due to location or geography. These

groups are possibly separate for the same reasons outlined for the north *S. glaucus* populations.

Hybridization

Identification of *Sclerocactus* taxa in the field has been described as challenging. Assignment of populations is based on morphological characters associated with a particular species. *Sclerocactus glaucus* is assigned when the population is in the correct geographical range and individuals in the population have straight spines. *Sclerocactus parviflorus* have some range overlap with *S. glaucus* and is identified when populations have individuals with characteristically hooked spines. Intermediate morphologies have been observed among *S. glaucus* populations that have raised questions about hybridization or character trait plasticity.

In order to uncover what is being observed in these populations, STRUCTURE analyses can be used for definitive answers. If the assigned morphological characters are not reliable in taxonomic identification, it is possible that populations have been misidentified. Misidentification of populations could misdirect conservation efforts and impede efforts to limit hybridization. Our research found that both Stage Coach Trail and Grand Junction Airport populations have been misidentified as *S. parviflorus* but are now known to be *S. glaucus* populations containing individuals with characteristics traditionally associated with *S. parviflorus* (Figures 8 and 9). The Grand Junction Airport population has minimal genetic signal from *S. parviflorus* indicating it is a pure *S. glaucus* population, composed of only the southern *S. glaucus* genetic signal. The data from Stage Coach Trail indicate that it is largely southern *S. glaucus* with some hybrid individuals with genetic signals from both the northern *S. glaucus* and *S. parviflorus.*

Representatives of individuals found in the Stage Coach Trail population are shown in Figure 9. However, one of these individuals has negligible *S. parviflorus* genetic signal (0.50%) while the other has substantial hybridization (40%). These individuals demonstrate that morphology is not an accurate indicator of hybrid individuals.

Figure 8: Individuals from the Grand Junction Airport population that have been identified as *S. parviflorus* based on spine morphology but are genetically *S. glaucus* with extremely low levels of *S. parviflorus* hybridization. Notice the characteristically hooked spines.

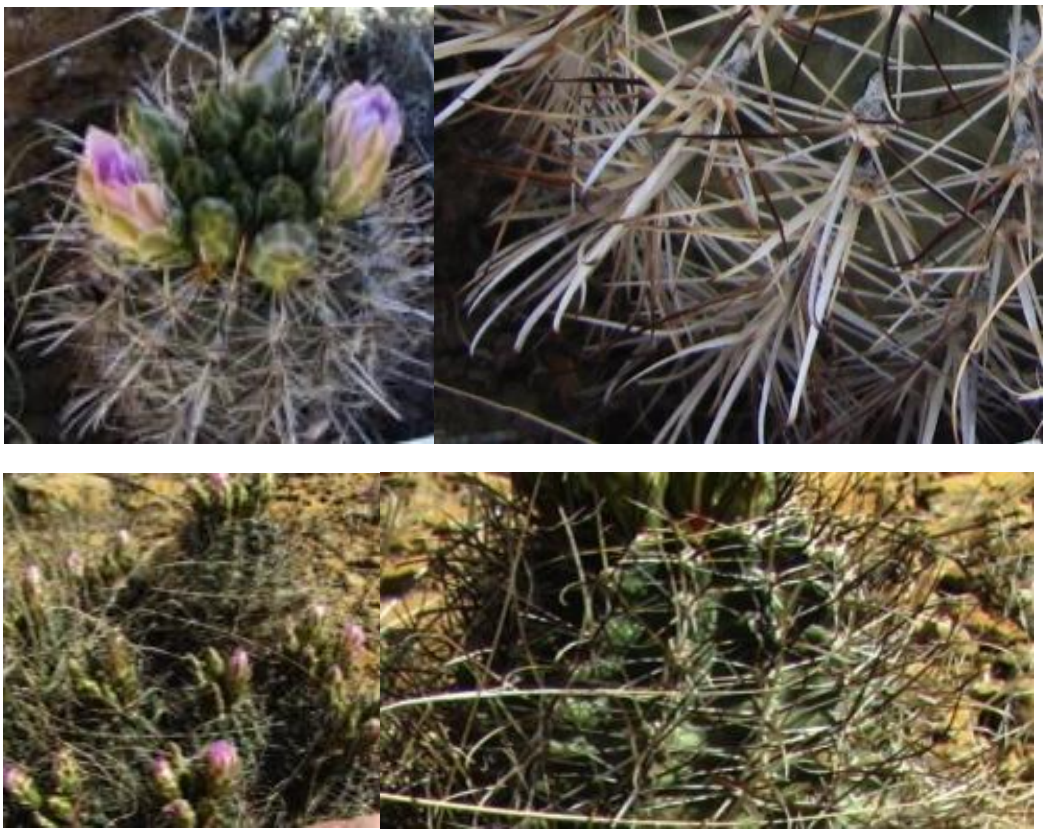


Figure 9: Comparisons of two individuals from Stage Coach Trail with significant differences of introgression from *S. parviflorus (*top: 0.50%, bottom: 40%) demonstrating morphology is not reflective of genotype.

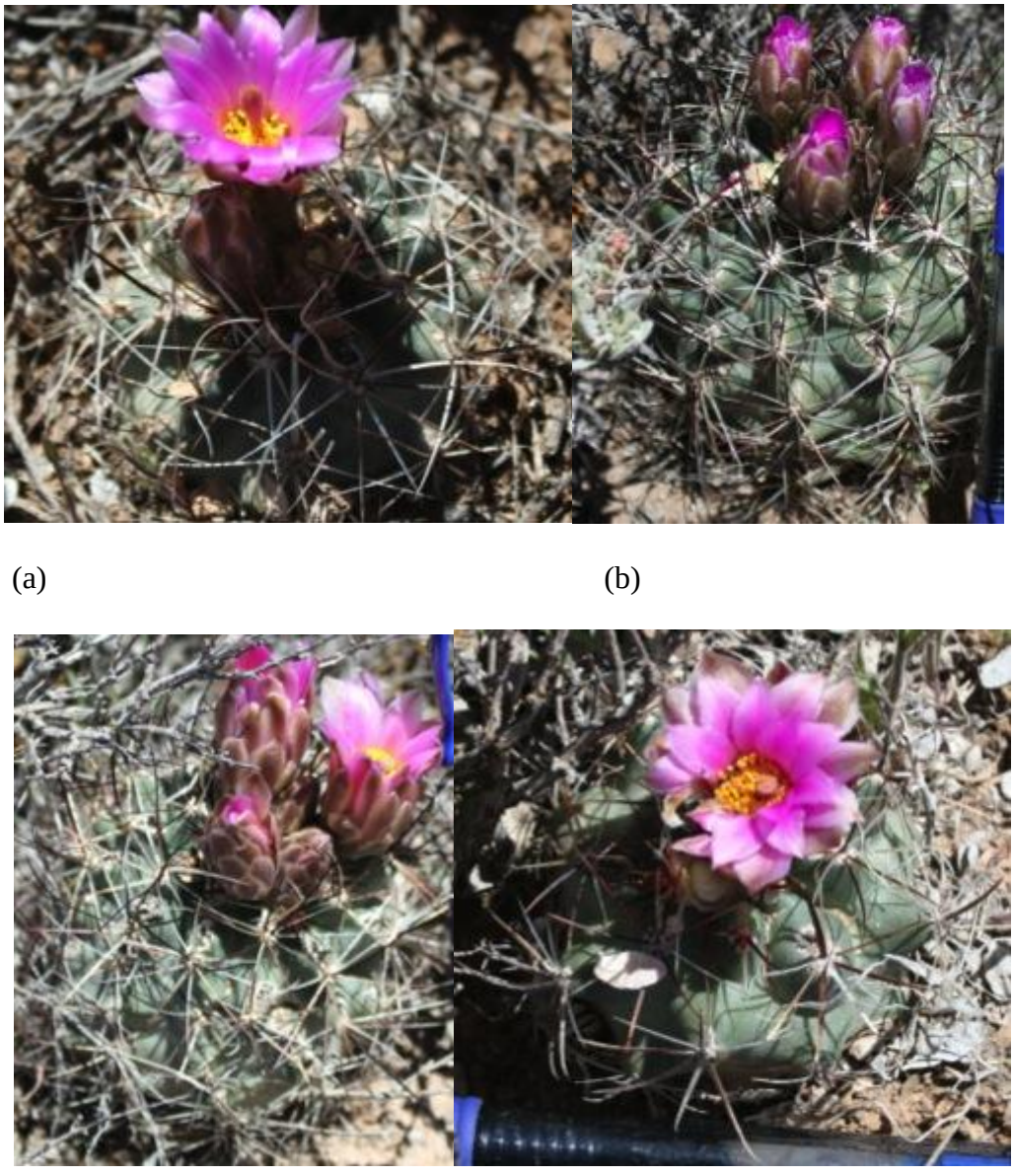
Analysis of the 28 *S. glaucus* populations has revealed that there is minimal introgression and hybridization between *S. glaucus* and *S. parviflorus.* While the division of the evolutionary groups appears to be geographically related, the locations of the hybrid individuals are not. Overall there are very few individuals with substantial (21) and minimal (27) hybridization that are spread among the 28 populations. South Shale Ridge, Atwell Gulch and Red Hill are north *S. glaucus* populations that have hybrid individuals. Interestingly, each one of these three populations is located in a different genetic group of the north *S. glaucus* populations. The northern *S. glaucus* populations

are located north east of Grand Junction while *S. parviflorus* populations do not appear to occur east of Grand Junction. This indicates there is possibly some sort of isolating barrier. Another possibility is that *S. parviflorus* pollen or seed has moved from one area to the other due to human movement. Urbanization, recreation, development and exploration in this area of Colorado could result in new genetic corridors allowing for genetic flow between these areas.

The south *S. glaucus* populations that appear to have hybrid individuals are Stage Coach Trail in the north, Powerline in the west and Dominguez Canyon, Cactus Park and Wells Gulch in the east. Wells Gulch is by far the population that has the heaviest influx of *S. parviflorus* genetic material. The pattern of gene flow from *S. parviflorus* into these populations is not clear based on their locations. In addition to the *S. parviflorus* genetic exchange, it may be of some use to note that there is more genetic material within *S. glaucus* populations moving from the north populations into the south populations. STRUCTURE indicates there are many more individuals in the south *S. glaucus* group with some north signal than there is south *S. glaucus* signal in the north groups. Suggesting possible human activities, which may be facilitating gene flow in this area would be speculation. Land managers may be able to add some additional insights as to activities that may be facilitating genetic material moving in and between these populations in this area.

The STRUCTURE data confirms that intermediate morphologies do not necessarily reflect the genome of a hybrid individual. Characters, such as hooked spines, historically designated for *S. parviflorus,* are not good indicators for determining species within a population (Figures 9 and 10). Although the data indicate the presence of gene

flow and hybrid individuals within *S. glaucus* populations, there are very few of these individuals and hence they are of minimal concern.



(a) (b)

(c) (d)

Figure10: Individuals #2, #3, #7, and #22 from the hybrid *S. glaucus* population Wells Gulch. These individuals show the highest introgression of *S. parviflorus* genetic signal at (a) 31%, (b) 56%, (c) 45% and (d) 35%, respectively.

Wells Gulch, which is the most heavily hybridized population, demonstrates that genotype is not driving spine morphology among *Sclerocactus* species. The individuals in Wells Gulch contain individuals with morphologies that would suggest that all members of this population are *S. glaucus* even though this population has undergone substantial hybridization with over half of the sampled individuals containing > 10% *S. parviflorus* genome (Figure 10). For these reasons, it is not prudent to assign species identifications to *Sclerocactus* populations in the Gunnison River and Colorado drainage system based on morphology alone. Figure 10 shows individuals from Wells Gulch with *S. parviflorus* genetic signal ranging from 31-56%. These individuals have typical morphologies found in this population and do not suggest that there is substantial hybridization.

The hybridization results lead to several suggestions for conservation of the threatened *S. glaucus.* Wells Gulch, which has substantial hybridization, should be isolated from other *S. glaucus* populations to minimize introgression of *S. parviflorus* to adjacent or nearby *S. glaucus* populations. Additionally, there are a few populations that are not of concern immediately but could present a threat if left unchecked. Cactus Park, Dominguez Canyon and Atwell Gulch each have minimal hybridization within the population, but many individuals in each population have *S. parviflorus* signal. With additional *S. parviflorus* introgression along with inbreeding, subsequent generations in these populations could experience increased levels of *S. parviflorus* genome. Something that was not expected is the number of *S. glaucus* population that had little to no *S. parviflorus* introgression. South Shale T-junction, Mile Post 68 North, Halfway House, ON1 East Basin, Grand Junction Airport, Gunnison River, Escalante Canyon 1 and 2 McCarty Bench each have extremely low levels of *S. parviflorus* hybridization and can

be considered pure *S. glaucus* populations. Devils Thumb, ON1 North Ridge and Whitewater Reeder Mesa have very low introgression in all but one individual.

Diversity

Endangered and threatened species are of concern to conservation biologists as a decline in both numbers of populations and population size can lead to decreases in genetic diversity. Natural and anthropogenic processes leading to smaller populations may lead to decreasing diversity and may hinder evolutionary processes (Etterson and Shaw 2001; Gomulkiewicz and Holt 1995; Willi et al., 2006). Although *S. glaucus* is often found in small populations that are isolated or fragmented, our research indicates that many of these populations are not genetically depleted. When considering populations of interest for conservation measures, heterozygosity (H) is of utmost importance. A heterozygosity of 1.0 indicates no shared alleles between individuals and therefore a high level of diversity. A value of 0 indicates that all individuals are identical and there is no diversity among them. Generally a value of 0.30 indicates moderate diversity and all populations of *S. glaucus* are above this value for both expected (H_e) and observed (H_o) heterozygosity (Table 3). The data for each population of *S. glaucus* indicate that none of the populations are genetically depauperate. It is of some interest to notice that both mean values of H_e and H_o were lower for than *S. glaucus* for both *S. parviflorus* (0.62 and 0.38, respectively) and *S. cloveriae* (0.55 and 0.35, respectively). As a general trend, there are less effective alleles than average alleles. This is to be expected as effective alleles are calculated using the inverse of the homozygosity. The number of effective alleles is the number of alleles it would take to produce the observed heterozygosity in the population. Smaller populations and rare species are subject to high

levels of genetic drift, so homozygotes tend to be more abundant in these populations. The largest differences between average and effective allele number in *S. glaucus* occur in Cactus Park, Dominguez Canyon and Reeder Mesa. Interestingly the mean numbers in *S. parviflorus* and *S. cloveriae* were lower for both average and effective alleles indicating there is more diversity within *S. glaucus* populations than there is in *S. parviflorus* populations.

Generally an inbreeding coefficient of 0.50 or lower is not considered to be of concern among plant populations, however values greater than 0.50 indicate a higher amount of inbreeding. Inbreeding can lead to inbreeding depression and a loss of genetic diversity, so populations with high F_{IS} values may be of less priority for conservation. The inbreeding data shows no excessive inbreeding in any population for any of the three species (Table 3). Among, *S. glaucus* the highest inbreeding is in Horse Mountain (0.46), Reeder Mesa (0.40) and Stage Coach Trail (0.39), which are located in the same general area. These numbers may indicate the presence of a reproductive barrier in the area. Other *S. glaucus* populations with moderately high inbreeding are the South Shale populations (0.34-0.39) (Table 3) in the north, which are geographically adjacent and could be considered perhaps as a single large population. Milepost 68 also in the north *S. glaucus* group has a moderately high F_{IS} of 0.38 (Table 3) but is located the farthest north in the Colorado River drainage, which may limit gene flow with other populations. Finally Atwell Gulch and Adobe Hills in the south *S. glaucus* group both have F_{IS} values of 0.35 (Table 2), but are not isolated from other populations.

The genetic distance between *S. glaucus* and *S. parviflorus* (F_{ST} 0.15) (Table 5) is large enough to infer that these species have diverged enough to be designated as distinct

and separate species. Porter et al. (2007) concluded the same using AFLP data. The F_{ST} results also suggest that *S. cloveriae* is not merely a New Mexico variety of *S. parviflorus*, but rather is distinctive enough from both *S. glaucus* and *S. parviflorus*, with an of F_{ST} 0.19 and 0.17 respectively, to maintain a separate species designation. Additionally, the low genetic distance within the north and south *S. glaucus* groups suggests that there is negligible divergence in the north and minimal divergence in the south. However, there is a significant divergence of the north *S. glaucus* found in the Colorado River drainage from the south *S. glaucus* found in the Gunnison River drainage.

Populations containing the highest number of heterozygotes and lowest inbreeding coefficient are of particular conservation interest. Such populations are less likely to suffer from inbreeding depression leading to decreasing diversity levels. Diversity levels and inbreeding levels should be considered along with *S. glaucus* population purity to establish where management efforts would be best directed. Table 5 outlines various populations that have large enough populations to warrant preferential conservation, and that have respectable levels of diversity and a low incidence of inbreeding. Management for the preservation of these natural populations would require land management in order to prevent disturbance development and other detrimental anthropogenic activities in the area where these populations occur. These populations should be targeted based on not only the low level of *S. parviflorus* introgression, but also because these populations have either a higher average number of alleles and/or higher observed heterozygosity, both of which contribute to diversity. An additional factor to consider when directing management efforts is the level of inbreeding. No *S. glaucus*

population exhibited high inbreeding levels, but lower levels of inbreeding are more desirable in order to maintain diversity levels within the species.

Conclusion

Attempts to preserve and manage threatened and endangered species are important to maintain genetic diversity that drives evolutionary processes. Ecological systems are in a delicate balance and the loss of species in any system may have devastating effects on other species within the system. Our data have uncovered some interesting patterns of hybridization and population structure of *S. glaucus* and *S. parviflorus.*

Bayesian cluster analysis in STRUCTURE and PCoA data suggest that *S. glaucus* and *S. parviflorus* are distinct and separate species and should be treated as such. Additionally, there are two unique groups of *S. glaucus* found in two different river drainages, which are distinct enough from each other that they should be managed as distinct and separate evolutionary units. The misidentification of Grand Junction Airport and Stage Coach Trail populations indicates that taxonomic identification of *S. glaucus* should not be based on morphology alone. *Sclerocactus parviflorus* populations do not seem to occur east of Grand Junction, which indicates there is a geographical distinction as to where the two species exist. *Sclerocactus* populations found in either the Gunnison River drainage or eastern Grand Valley and Colorado drainages have been found to be only *S. glaucus.* Therefore any *Sclerocactus* population found in either of these areas should be considered and managed separately as north or south *S. glaucus.* In order to preserve these unique genetic units, management should avoid relocating individuals or populations from one region to another.

Individuals in *S. glaucus* populations with a large *S. parviflorus* genetic signal could be targeted for removal so they are no longer able to contribute *S. parviflorus* genetic material to the species. The population showing the most hybridization, Wells Gulch, should be isolated from other *S. glaucus* populations to minimize the possibility of introgression into adjacent populations. Populations of *S. glaucus* with minimal *S. parviflorus* genetic signal along with greatest diversity levels should be given conservation priority. If management resources allow, almost all populations of *S. glaucus* are important and should be preserved, monitored, maintained and managed.

CHAPTER IV

CHLOROPLAST DNA

Introduction

Sclerocactus glaucus is a federally threatened species that has a small range over four counties near Grand Junction, Colorado. *Sclerocactus glaucus* habitats are located in areas of gas and oil exploration as well as open range cattle and recreational areas. Conservation biologists are concerned that *S. glaucus* could be facing extinction not only from habitat disturbance but also through hybridization with *Sclerocactus parviflorus*, which is a neighboring closely related species. Hybridization can occur when a flower of one species is pollinated by another species creating hybrid seed. A hybrid seed will have 50% of the nuclear genome from each parent and the chloroplast genome of the maternal parent. This is because chloroplasts are uni-parentally inherited from the seed parent (Provan et al. 2001). Pollen can be water, wind or insect transferred, but with increasing exploration and cattle grazing in *Sclerocactus* habitat, it is possible that *S. parviflrous* pollen and seeds are being transferred into *S. glaucus* populations by the opening of new corridors for increased gene flow and by the physical presence of human activity in the area (Tepedino et al. 2010). Additionally, seed or plants from one species may transfer and germinate in a population of a closely related species. If an *S. parviflorus* individual grows within an *S. glaucus* population it could lead to hybridization within that population.

Anthropogenic seed, pollen, and plant transfer between populations is of concern to conservation biologists. With resource exploration, oil drilling and cattle grazing in *Sclerocactus* habitat, it is possible that new corridors for gene flow have opened (Rhymer and Simberloff 1996). Corridors could be created from habitat disturbance such as roadways created for vehicles or from the physical breakdown of isolating barriers that previously prevented movement of pollinators between populations (Tepedino et al. 2010). Additionally, pollen and seed movement may be directly facilitated by human activity. Tire treads on vehicles, cattle hooves and fur, or socks and shoes of humans could be potential contributors to the movement of seeds between populations.

Analysis of chloroplast DNA can be useful in order to determine if genetic signals originated from pollen or seed. Chloroplasts are maternally inherited from the seed parent and this can give useful information as to how hybridization is occurring (Lian et al. 2008; Ouborg et al. 1999). For the following analyses using chloroplast DNA (cpDNA), the expectation is to see genetic signal corresponding to the species identified within the populations. If there is cpDNA signal from *S. parviflrous* among *S. glaucus* populations, or vice versa, it may indicate seed transfer is occurring, not just pollen flow. All the individuals chosen for this portion of the project were selected based on a mixed genetic signal of either *S. parviflrous/ S. glaucus,* north/ south *S. glaucus* or *S. parviflorus /S. cloveriae,* based on genetic structure from nuclear microsatellite analyses (Chapter III). The analysis of cpDNA signal will be used to make conclusions as to which species is the maternal parent in the hybrid individuals. It is possible to determine the origin of an individual with both nuclear DNA and the species identity of the maternal lineage. If an individual has chloroplast DNA from another species it could signify that there has been

seed or plant transfer into a population. This information may assist land managers when they are determining management strategies to preserve the integrity of *S. glaucus* populations.

Methods

DNA Extraction

The DNA used for the following procedures were extracted using the methods outlined in Chapter II.

Chloroplast PCR Sequencing

Sixteen general cpDNA primers (Shaw et al. 2005) were tested with 2-4 individuals each of *S. glaucus, S. parviflrous* and *S. cloveriae.* The primers tested were *trnK(UUU)x1-rps16x2f2, trnD(GUC)-F-trnT(GGU), trnL(UAG)-rpl32f, rpl32-R-ndhF, trnQ(UUG)-rps16x1, trnT(GGU)-R-psbD, trnT(UGU)F(TabA)-5'trn(UAA)-R-TabB, trnV(UACx2-ndhC, atpH-atpI, psbJ-petA, psbE-petL, trnF(GGA)-trnL-5(UAA)f, 5'TrnL(UAA)R-trnT(TabA), trnC-rpoB, psbA-trnH, trnS-5'trnG* (Shaw et al. 2005). PCR amplifications were carried out with 20 µL reactions with1.0 µL genomic DNA, 1.0 µL non-tagged primer (5µM)**,** 1.0 µL tagged primer (0.50 µM)**,** 1.0 µL dNTP mixture (at 2.5 mM)**,** 0.30 µL Taq polymerase (Promega, Madison, WI, USA), 4.0 µL GoTaq Flexi Buffer (Promega, Madison, WI, USA), 11.7µL dH_2O and 1.0 µL $MgCl_2$. A master mix was prepared with enough reagents for all reactions and was then loaded into 96 well plates**.** PCR amplification was carried out on an Eppendorf Mastercycler proS (Hamberg, Germany). An initial 5 minute denaturing step at 80º C was followed by thirty amplification cycles of 1 min denaturing at 95º C, 1 min denaturing at 50º C, 4 min denaturing at 65º C with a 2 ramp, followed by a 5 minute extension at 65º C (Shaw et

al. 2005). Amplification products were verified visually on a 1% agarose gel. Of the 16 primers tested, 11 showed positive amplification.

Amplified PCR products were cleaned using an ExoSAP-IT procedure. This procedure used hydrolytic enzymes Exonuclease I (Exo I) and FastAP^{TM} Thermosensitive Alkaline Phosphatase or Shrimp Alkaline Phosphatase (SAP) to remove any residual primer sequences or unincorporated nucleotides. Following PCR 5 µL of product was mixed with 0.50 µL of Exo I and 1 µL of SAP. The mixture was then placed in the Eppendorf Mastercycler proS (Hamberg, Germany) for a 30 minute cycle, which consisted of an incubation at 37^{o}C for 15 minutes followed by a cycle, which stopped the reaction by heating the mixture to 85^{o}C for 15 minutes.

Fluorescent cycle sequencing was performed using a dye terminator sequencing reaction. These reactions incorporated a dye that caused termination of the sequence that was replicated whenever a fluorescently labeled nucleotide was incorporated into the sequence. The reaction volumes were approximately 10 µL with 2 µL 5X dilution Buffer, 0.33 µL Big Dye III, 0.80 µL clean PCR product, 0.50 µL primer and 6.4 µL water. The reactions mixtures were placed in the Eppendorf Mastercycler proS (Hamberg, Germany) for an initial temp of 96^{o}C for 1 minute, followed by 30 cycles of 96^{o}C for 15 seconds, 50^{o}C for 20 seconds, 60^{o}C for 4 minutes and then incubation at 4^{o}C as a holding temperature.

Analysis

Fluorescently labeled PCR products were analyzed on an Applied Biosystems 3130 Genetic Analyzer at Arizona State University. The Applied Biosystems 3130 Genetic Analyzer created a consensus sequence for each reaction by piecing together the

various overlapping sequences with the incorporated fluorescent tags. These sequences were then analyzed for variability between the test samples. SEQUENCHER ver. 5.0 (Gene Codes Corporation) was used to analyze the sequences for variable sites in the sequence. Based on the analyses, two variable regions, *trnF(GGA)-trnL-5(UAA)f* and *trnC-rpoB,* were chosen for detailed data collection. Both strands from each of these cpDNA regions were sequenced and assembled in SEQUENCHER ver. 5.0 (2011).

SEQUENCHER ver. 5.0 (2011) was used to visualize the cpDNA sequences for all 77 individuals chosen for these analyses. SEQUENCHER presents a visual representation of the sequence code and allows resolution of ambiguities in the nucleotide sequence and also allows visual conformation of variability in the cpDNA sequences.

The cleaned sequences were then transferred into Se-Al ver. 2.0 (Rambaut 2007) for alignment of sequences and trimming for all 77 individuals. The sequences were then concatenated and the gaps were coded by hand.

DnaSP ver. 5.10.01 (Librado and Rozas 2009) was used to calculate chloroplast DNA diversity measures within and between *S. glaucus, S. parviflrous* and *S. cloveriae*. *Sclerocactus glaucus* was further divided into subpopulations for the diversity analyses based on STRUCTURE and Principle Coordinate Analysis of nuclear microsatellites (see Chapter III), which suggests that these two groups are genetically distinct from one another and should be treated as such. Additionally, the individual SSR6 from the north *S. glaucus* population South Shale Ridge, which was genetically identified as 87% *S. parviflrous*, was removed for some analyses. The diversity statistics reported were number of individuals sampled (N), number of haplotypes without gaps (Hp0), number of haplotypes with gaps (Hp), nucleotide diversity (Pi) and sequence lengths (Seq lgth) with

and without gaps. The number of pairwise nucleotide differences between populations (K_{XY}), the genetic differentiation index (G_{ST}), the fixation index (F_{ST}) and the average number of nucleotide substitutions per site between populations (D_{XY}) were measured.

MrBayes ver. 3.2 (Ronquist et al. 2011) was used to calculate Bayesian inference data to generate the phylogenetic trees. Two partitions of data were run simultaneously using the sequence data with the GTR+Gamma+invariants model. The gaps were analyzed using the standard model. A run length of 5,000,000 generations was used, saving every 1000^{th} tree. A burnin of 25% was used, and therefore the first 1250 trees saved were discarded. MrBayes (Ronquist et al. 2011) then summed the remaining 3750 trees to make a consensus tree.

Raw data was used to generate an unrooted phylogenetic tree was generated by FigTree ver. 1.3.1 (Rambaut 2009). Posterior probabilities are labeled on the branches indicating the frequency that each branch was resolved in the 3750 trees. A value of 1.0 indicates a branch was resolved in 100% of the trees, and is the highest level of support for a branch on a consensus tree.

A haplotype network was generated by TCS ver. 1.21 (Clement et al. 2000) using the Se-Al sequence alignment data and gaps were treated as a 5th state. The haplotype figure represented variation in the *trnF(GGA)-trnL-5(UAA)f* and *trnC-rpoB* chloroplast regions. The haplotype network (Clement et al. 2000) diagram was rendered as a figure using Microsoft Word® for Mac 2011 to clearly indicate haplotypes and mutational steps. Each branch indicates an inferred mutational step and each circle indicates an observed haplotype (colored circle) or inferred haplotype (white circles).

Results

Diversity

Nucleotide diversity statistics from cpDNA data within each of the *Sclerocactus* species groups is shown in Table 6. There were 77 total individuals sampled with data reported for 6 subgroups; *S. glaucus* (S), *S. glaucus* (N), *S. glaucus* (all), *S. glaucus* (no SSR6), *S. parviflorus* and *S. cloveriae.* The number of haplotypes without gaps (Hp0) was highest in all *S. glaucus* populations (4) and the lowest in north *S. glaucus* (exc SSR6) (1). The number of haplotypes with gaps (Hp) was highest for all *S. glaucus* (6) and the lowest number in north *S. glaucus* without individual SSR6 (1). The highest nucleotide diversity (Pi) was found in *S. cloveriae* (0.00036) and lowest was found in north *S. glaucus* (0.0000), which had no polymorphisms in the cpDNA.

Table 6. Nucleotide diversity of cpDNA within each of the *Sclerocactus* species groups

Species Group	N	Hp0	Hp	Pi	Seq lgth	Seq lgth
S. glaucus (S)	41	2	4	0.00006	1874	1870
S. glaucus (N)	17	1	2	0.00000	1874	1871
S. glaucus (all)	62	4	6	0.00010	1874	1870
S. glaucus (no SSR6)	61	3	1	0.00005	1874	1870
S. parviflorus	12	3	4	0.00025	1874	1873
S. cloveriae	3	2	2	0.00036	1874	1874

Number of individuals sampled (N), number of haplotypes without gaps (Hp0), number of haplotypes with gaps (Hp), nucleotide diversity (Pi) and sequence lengths (Seq lgth) with and without gaps.

Pairwise diversity statistics were calculated between *S. glaucus* (S), *S. glaucus* (N), *S. glaucus* (all), *S. glaucus* (no SSR6), *S. parviflorus* and *S. cloveriae* (Table 7). The number of pairwise nucleotide differences between populations (K_{XY}) was the greatest between south *S. glaucus* and *S. parviflrous* (2.9800) and lowest between south *S.*

glaucus and north *S. glaucus* without SSR6 (0.04900). The genetic differentiation index (G_{ST}) was the highest between *S. parviflorus* and north *S. glaucus* (0.68000), and was the lowest between all population of north *S. glaucus* (exc SSR6) and south *S. glaucus* (-0.00790), all population of *S. glaucus* and north *S. glaucus* without SSR6 (-0.00700), and south *S. glaucus* and north *S. glaucus* without SSR6 (-0.00790). The highest fixation index (F_{ST}) was between *S. parviflorus* and north *S. glaucus* (0.92000), and the lowest was between south *S. glaucus* and north *S. glaucus* without SSR6 (-0.00150). Negative G_{ST} and F_{ST} values are due to software idiosyncrasies and can be assumed to be zero (Humphries and Winker 2011). The average number of nucleotide substitutions per site between populations (Dxy) was the highest between all of the subpopulations of *S. glaucus* (all, north, north without SSR6 and south) and *S. parviflrous* (0.00160), and was the lowest between south *S. glaucus* and north *S. glaucus*, and north *S. glaucus* and north *S. glaucus* without SSR6 (0.00003).

Table 7. Pairwise diversity statistics between *Sclerocactus glaucus* all (*no SSR*), *S. glaucus* south, *S. glaucus* north, *S. glaucus* north excluding SSR6 (*S. parviflorus* Individual), *S. parviflorus*, and *S. cloveriae* populations for cp DNA analysis

Population 1	Population 2	K_{XY}	G_{ST}	F_{ST}	D_{XY}
S. glaucus (S)	*S. glaucus (N)*	0.59	0.0120	0.03	0.00003
S. glaucus (S)	*S. parviflorus*	2.98	0.5900	0.90	0.00160
S. glaucus (S)	*S. cloveriae*	2.40	0.3900	0.84	0.00130
S. glaucus (S)	*S. glaucus (N exc SSR6)*	0.10	-0.0079	-0.02	0.00006
S. glaucus (N)	*S. parviflorus*	2.90	0.6800	0.92	0.00160
S. glaucus (N)	*S. cloveriae*	2.30	0.6300	0.86	0.00130
S. glaucus (N)	*S. glaucus (N exc SSR6)*	0.05	0.0110	0.01	0.00003
S. glaucus (all pops)	*S. parviflorus*	2.90	0.5200	0.89	0.00160
S. glaucus (N exc SSR6)	*S. parviflorus*	3.00	0.5700	0.90	0.00160
S. glaucus (all exc SSR6)	*S. parviflorus*	2.97	0.5700	0.90	0.00160
S. glaucus (all pops)	*S. cloveriae*	2.40	0.3000	0.82	0.00130
S. glaucus (N exc SSR6)	*S. cloveriae*	2.40	0.3400	0.84	0.00130
S. glaucus (all exc SSR6)	*S. cloveriae*	2.38	0.3400	0.84	0.00127
S. parviflorus	*S. cloveriae*	1.30	0.2100	0.55	0.00067

Number of pairwise nucleotide differences between populations (K_{XY}), genetic differentiation index (G_{ST}), fixation index (F_{ST}), average number of nucleotide substitutions per site between populations (D_{XY}).

Phylogenetics

A strongly supported non-rooted phylogenetic tree based on the MrBayes results was generated (Figure 11). Posterior probability values ranged from 0.8400-0.9999. The *Sclerocactus* species are color coded to parallel the colors used in Chapter III for both the STRUCTURE diagram (Chapter III, Figure 3) and the PCoA (Chapter III; Figure 7). However, the phylogenetic tree (Figure 11) and the haplotype diagram (Figure 12) in this chapter have *S. cloveriae* coded in yellow. One individual from a north *S. glaucus* population, South Shale Ridge #6, has an S. *parviflrous* cpDNA haplotype. Structure analyses showed that SSR6 has an 87% parviflrous genetic signal based on microsatellite data.

Haplotypes

The haplotype network (Figure 12) shows mutational steps (including insertions and deletions) for south *S. glaucus* (blue), north *S. glaucus* (green), *S. parviflrous* (red) and *S. cloveriae* (yellow) populations. Each circle indicates an observed (colored circle) or inferred (white circle), cpDNA haplotype. Between the *S. glaucus* populations and the *S. parviflrous* populations there are four inferred mutational steps. There is a single *S. glaucus* individual (SSR6) that has an S. *parviflrous* haplotype. Additionally, *S. cloveriae* (yellow) has distinct variability in the chloroplast genome and is separated from *S. parviflrous* populations. The number of individuals assigned to each haplotype is given in Table 8.

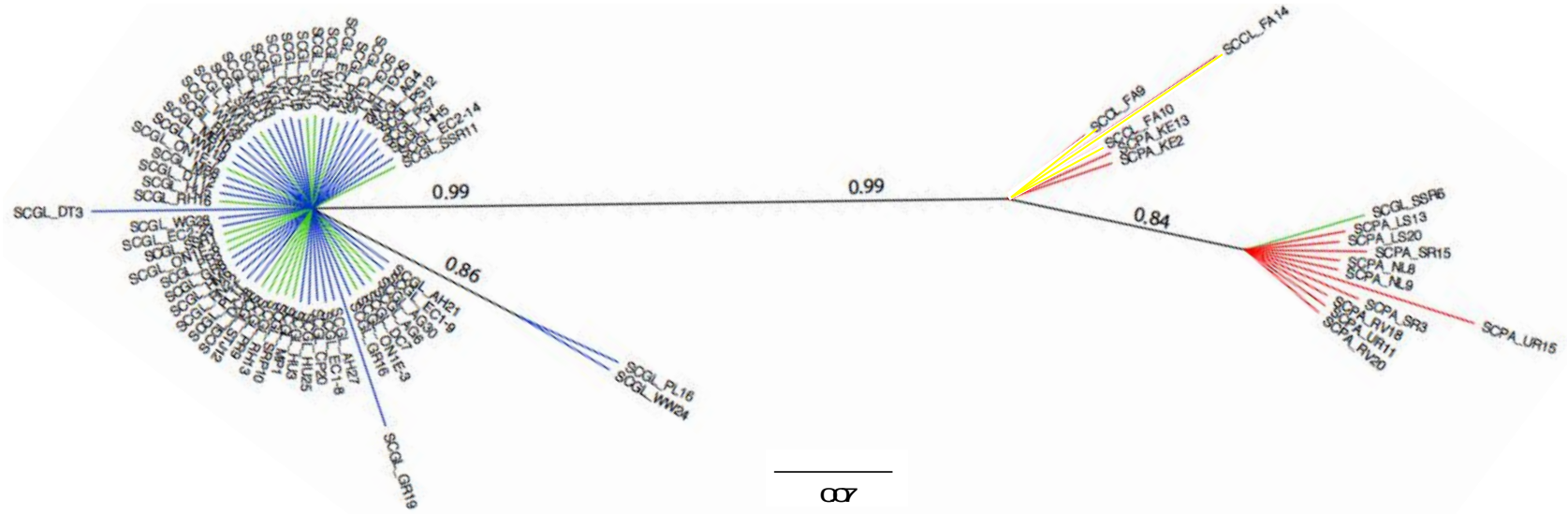


Figure 11: An unrooted Bayesian phylogenetic tree generated by FigTree (Rambaut 2009) showing strong support for variation in the *trnF(GGA)-trnL-5(UAA)f* and *trnC-rpoB* chloroplast regions among south *S. glaucus* (blue), north *S. glaucus* (green), *S. parviflrous* (red) and *S. cloveriae* (yellow). One individual from a north *S. glaucus* population (SSR6) identifies with *S. parviflrous.*

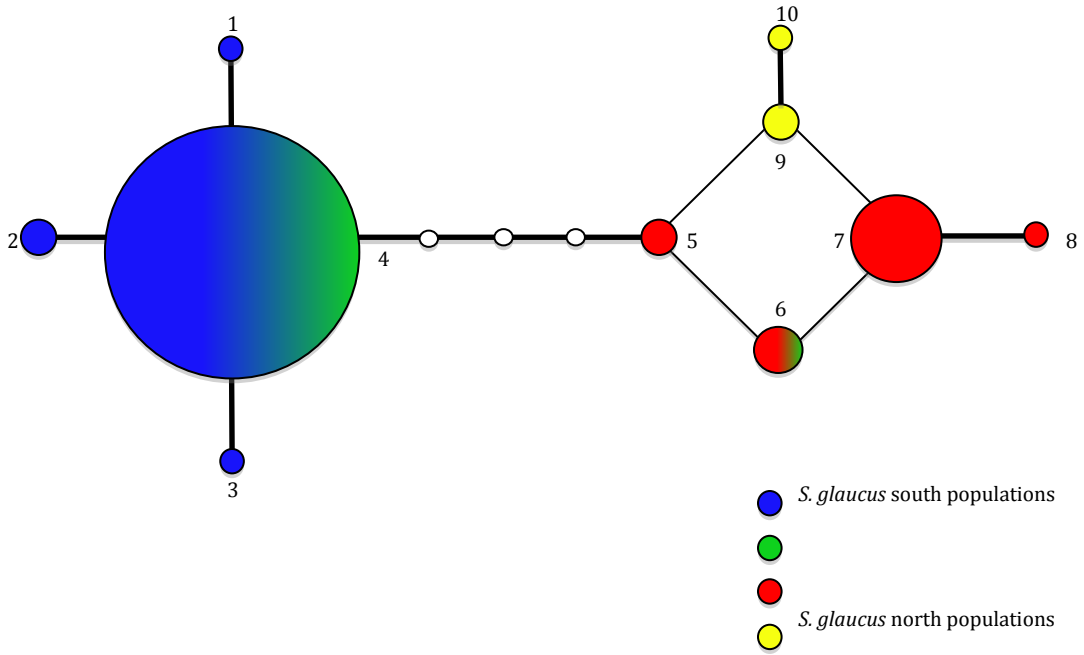


Figure 12: Haplotype diagram showing variation in the *trnF(GGA)-trnL-5(UAA)f* and *trnC-rpoB* chloroplast region for the south *S. glaucus* (blue), north *S. glaucus* (green) *S. parviflrous* (red) and *S. cloveriae* (yellow) populations. Each branch indicates an inferred mutational step. Each circle indicates an observed (colored circle) or inferred (white circle) cpDNA haplotype. There is a single north *S. glaucus* individual (SSR6) that has *S. parviflrous* cpDNA haplotype.

Table 8. Number of individuals and species assignment relating to the haplotype diagram (Figure 12)

Circle number	Species	N
1	*S. glaucus (south)*	1
2	*S. glaucus (south)*	2
3	*S. glaucus (south)*	1
4	*S. glaucus (south)*	37
	S. glaucus (north)	20
5	*S. parviflorus*	2
6	*S. parviflorus*	2
	S. glaucus (north)	1
7	*S. parviflorus*	7
8	*S. parviflorus*	1
9	*S. cloveriae*	2
10	*S. cloveriae*	1
	total	77

Circle number correlates to the labeling of the circles in Figure 12, N is the number of individuals in each haplotype group.

Discussion

Chloroplast genomes are highly conserved and within a genus will show little variability. Chloroplasts are maternally inherited and can provide information about how gene flow is occurring. Using hybrid individuals chosen based on genetic structure, it is possible to determine where the chloroplast genome of an individual originated. If an individual has chloroplast DNA from another species it could signify that there has been seed or plant transfer into a population. Although entire plant transfer is not feasible for most plant species, cacti have the ability to be uprooted, transferred and reestablish in a different area. In the case of either seed or plant transfer between populations of different species, a hybrid individual would have the chloroplast genome of the alternate species.

Microsatellite data has indicated that there is some hybridization between *S. glaucus* and *S. parviflorus,* but this data gives little information as to where the hybrid genetic signals originated. Pollen movement is facilitated naturally by pollinators and is one way for congeners to hybridize. Of higher concern is *S. parviflorus* seed or entire plant transfer into populations of *S. glaucus.* Chloroplast DNA analysis indicates that of the *S. glaucus* 62 individuals sampled for cpDNA analyses, one *S. parviflorus* haplotype is located within an S. *glaucus* population. This plant (SSR6) identifies with a group that has six inferred mutational steps from other individuals in the same population. The chloroplast genetic signal from SSR6 indicates that it is identical to a *S. parviflorus* population that was sampled in New Mexico. The 87% *S. parviflorus* signal in SSR6 suggests that it is either a pure *S. parviflorus* individual or a direct descendant of a pure *S. parviflorus*. The population that SSR6 is located in is actually three populations in very close proximity. There were two other individuals from SSR and four additional individuals from the adjacent populations sampled for cpDNA, none of which had an *S. parviflorus* chloroplast haplotypes. The sampling for this study was minimal and not all hybrid individuals were analyzed. Discovering one individual in an *S. glaucus* population indicates that a much wider sampling of individuals should be carried out to ensure no additional *S. parviflorus* individuals are located in South Shale Ridge or surrounding populations. Since only one individual was found with a cpDNA chloroplast from another species, this indicates that the majority of hybridization is occurring via pollen rather than seed or plant movement from human activity. Pollination leading to hybridization may be due to disturbance or developments creating previously absent

corridors for pollinator movement. Therefore, there should be research carried out on the pollinators as well as a wider sampling for cpDNA variation.

Porter et al. (2000) used chloroplast data from the *trnL* intron and the *trnL-trnF* intergenic spacer to address some of the questions surrounding the taxonomy of *Sclerocactus* species. Their results showed no resolution between *S. glaucus, S. parviflorus* and *S. cloveriae.* The chloroplast data in this study strongly supports that *S. glaucus* and *S. parviflorus* are distinct species. Nucleotide diversity (Pi) within the chloroplast regions *trnF(GGA)-trnL-5(UAA)f* and *trnC-rpoB* appears to be slight, but because the chloroplast genome is highly conserved, low diversity is expected (Table 6). Additionally, the sample size was small in both *S. parviflorus* and *S. cloveriae,* so these values would probably decrease given larger sample sizes. The single *S. parviflorus* (SSR6) individual in the *S. glaucus* population appears to be artificially decreasing genetic divergence measures between the species (Table 7), and bringing down the values for the average number of nucleotide substitutions per site between populations (D_{XY}). However, removal of SSR6 from the analyses shows a more accurate portrayal of the genetic distance. Between the various subpopulations of *S. glaucus* there is minimal divergence. Between *S. glaucus* and *S. parviflorus* the pairwise genetic distance is much greater indicating they are distinct species. These findings support that not only the nuclear genome in these species have diverged, but also the highly conserved chloroplast genome. The phylogenetic tree strongly reinforces that *S. glaucus* is related to, but is sister species to both *S. parviflorus* and *S. cloveriae* (Figure 11). The haplotype diagram also supports these findings (Figure 12). A mixture of north and south *S. glaucus* individuals creates a large group of 62 individuals. This diagram shows that north and

south *S. glaucus* populations are not distinguishable. There are three additional *S. glaucus* haplotypes that have one mutation each compared to the large north and south *S. glaucus* group.

There are four inferred mutational steps between *S. glaucus* and the closest relatives found in *S. parviflorus,* which are from Kings Estate. Interestingly Kings Estate is the closest sampled *S. parviflorus* population to populations of *S. glaucus.* There is an additional mutation that distinguishes the next group of *S. parviflorus* individuals, which contains the SSR6 individual from the north *S. glaucus* population. The number of mutational steps from *S. glaucus* to the group with chloroplast DNA identical to SSR6 gives further support to the suggestion that this individual is an *S. parviflorus* individual located within an *S. glaucus* population and should be removed as soon as possible.

The chloroplast data surrounding *S. cloveriae* does not, on its own, give support to *S. cloveriae* being a distinct and separate species. Both the phylogenetic tree and the haplotype figures (Figures 11 and 12) show that *S. cloveriae* is not distinct enough in the chloroplast genome to be separated from *S. parviflorus.* The genetic diversity statistics show an intermediate level of pairwise divergence when compared with the values between *S. glaucus* and *S. parviflorus* (Table 7). However, there was only one population of *S. cloveriae* sampled and further sampling of *S. cloveriae* may give more clarity to if it is a separate species. Additionally, *S. parviflorus* was not extensively sampled overall and the sampling was done over a very large geographical area. Additional sampling of both *S. parviflorus* and *S. cloveriae* could resolve the question of whether these two species are distinct enough to be designated as separate species, or if *S. cloveriae* should be considered a New Mexico variety of *S. parviflorus.*

Conclusion

The chloroplast DNA data supports the distinction between *S. glaucus* and *S. parviflorus.* The genetic distance measures for cpDNA give further support to the STRUCTURE and PCoA results from Chapter III. Individual SSR6 is from the north *S. glaucus* population South Shale Ridge, but clearly has an *S. parviflorus* chloroplast haplotype. This indicates that this individual germinated from *S. parviflorus* seed or was a transplant individual from an S. *parviflorus* population. This individual may be responsible for some of the increased *S. parviflorus* signal observed in the South Shale Ridge populations from the STRUCTURE analysis. This individual should be located and removed from this population in order to halt any further introgression of *S. parviflorus.* Additionally, there should be further cpDNA analyses of this population to ensure there are no additional local hybridization threats within South Shale Ridge and the populations closely associated with it. The data from the chloroplast analysis of *S. glaucus, S. parviflrous* and *S. cloveriae* shows that (a) *S. glaucus* and *S. parviflorus* are distinct species (b) north and south *S. glaucus* groups are not divergent in relation to the chloroplast genome and (c) there is one known *S. parviflorus* individual in an *S. glaucus* population which should be removed in order to minimize subsequent hybridization within that population.

CHAPTER V

SUMMARY

Sclerocactus glaucus (K. Schumann) L.D. Benson (Cactaceae), the Colorado hookless cactus, is a rare species that is currently listed as threatened and protected under the Endangered Species Act. *Sclerocactus glaucus* is located in a small range in western Colorado spanning four *counties* around Grand Junction (USFWS 2010). *Sclerocactus* habitat disturbance is of high concern to conservationists as this area is subject to oil and gas exploration, urbanization, trampling from livestock and off road vehicle damage (USFWS 2007).

Traditionally, *Sclerocactus* have been identified based on morphological characteristics; straight spines have been associated with *S. glaucus* and hooked spines have been associated with *S. parviflorus,* which is a close relative with a nearby distribution (Heil and Porter 2004). However, within *Sclerocactus*, taxonomy based on morphology has been unclear (Porter et al. 2000*).* Previous genetic studies of *Sclerocactus* using chloroplast DNA yielded no clear resolution as to the genetic distinctions between *S. glaucus, S. parviflorus* and other closely related species of *Sclerocactus* (Porter et al. 2000). Possible misidentification of wild populations and unresolved genetic research has led to the need for further study. Intermediate morphologies in wild populations have led to fears of hybridization between these two species. Hybridization between *S. glaucus* and *S. parviflorus* may lead to genetic

swamping and ultimately extirpation of *S. glaucus* if there is a high level of gene flow from *S. parviflorus.* Extinction due to hybridization is of great concern if small populations are subject to an infiltration of genetic material from a closely related species (Rhymer and Simberloff 1996).

In order for land managers to effectively manage the remaining *S. glaucus* populations, there needs to be clarification of the relationship between *S. glaucus* and *S. parviflorus* as well as knowledge on purity of populations. Populations of *S. glaucus* that would warrant conservation priority would be populations with minimal introgression from *S. parviflorus,* with high diversity and low inbreeding. The questions addressed in this genetic study are (a) are *S. glaucus* and *S. parviflorus* distinct and separate species, (b) are *S. glaucus* and *S. parviflorus* hybridizing, (c) to what extent are *S. glaucus* and *S. parviflorus* hybridizing, (d) are there pure populations of *S. glaucus,* (e) are there hybrid populations that need to be confined, (f) what levels of diversity are there in populations of *S. glaucus,* (g) are there *S. parviflorus* individuals within populations of *S. glaucus?*

Morphological Distinctiveness

Previous conventions held that *S. glaucus* individuals had strait spines and *S. parviflorus* had hooked spines and this was the easiest way to identify the two species in the field. Field botanists observed intermediate morphologies bringing about hybridization concerns. Based on the data from this genetic investigation, morphology is not indicative of species identity in these two species. There are populations of pure glaucus that have hooked spines and hybrid populations with straight spines (Table 9).

Table 9. Populations that should be considered for conservation priority based on individual numbers sampled within the population, number of effective alleles, observed heterozygosity and level of inbreeding.

Species	Location	N	A	Ho	FIS
Devils Thumb	Colorado (S)	18	6.69	0.47	0.31
Ravens Nest	Colorado (S)	30	8.62	0.58	0.18
Escalante Cyn 1*	Colorado (S)	13	5.46	0.48	0.28
Escalante Cyn 2*	Colorado (S)	30	7.46	0.53	0.24
Picnic Site	Colorado (S)	30	7.46	0.5	0.26
McCarty Bench*	Colorado (S)	14	6.92	0.53	0.20
Gunnison River*	Colorado (S)	30	7.92	0.5	0.22
Reeder Mesa	Colorado (S)	29	9.08	0.42	0.40
GJ Airport*	Colorado (S)	15	7.00	0.48	0.20
Pyramid	Colorado (N)	30	6.85	0.47	0.24
S. Shale Ridge Pond	Colorado (N)	30	6.85	0.4	0.39
S. Shale Ridge	Colorado (N)	24	7.85	0.45	0.35
S. Shale Ridge T-Junction	Colorado (N)	23	7.15	0.43	0.34
Red Hill	Colorado (N)	26	6.69	0.46	0.25
ONIE/R	Colorado (N)	28	7.62	0.49	0.28
Milepost 68*	Colorado (N)	19	5.23	0.39	0.38

Microsatellite data using 13 variable loci were used to analyze populations of *S. glaucus, S. parviflorus* and a third sample from *S. cloveriae.* The Bayesian clustering analysis program STRUCTURE and principle component analysis showed three clear divisions that included north *S. glaucus* populations, south *S. glaucus* populations, and *S. parviflorus* grouped with the one *S. cloveriae* population (Figure 3 and 7). STRUCTURE and PCoA data show that *S. parviflrous* and *S. glaucus* are distinct and separate species, but also that the north and south *S. glaucus* groups are distinct and separate from each other. Two populations that had been previously designated as *S. parviflorus* based on morphology (Grand Junction Airport and Stage Coach Trail) resolved genetically as *S.*

glaucus populations. The misidentification of two populations shows that taxonomic identification of *S. glaucus* should not be based on morphology alone. Additionally, the population with the most introgression from *S. parviflorus* had no observed intermediate morphologies (based on photographs of sampled individuals), which gives support to the conclusion that morphology is not necessarily indicative of hybrid ancestry.

Hybridization

Sclerocactus glaucus and *S. parviflorus* have adjacent ranges and species identification is assigned based on geographical range and spine morphology (Heil and Porter 2004). Intermediate morphologies have raised questions about hybridization or character trait plasticity in *Sclerocactus* populations. Microsatellite data show that among *S. glaucus* populations there is minimal introgression from or hybridization with *S. parviflorus.* The division of the evolutionary clusters seems to be related to geographical location, but there is no clear pattern relating to the locations of the hybrid individuals. Of the 664 *S. glaucus* individuals sampled, there are relatively few individuals with substantial (21) and minimal (27) hybridization. These 48 hybrid individuals are spread out across many of the *S. glaucus* populations with usually only 1-3 hybrids per population. There is gene flow from *S. parviflorus* populations and there are hybrid individuals within *S. glaucus* populations, but there are relatively few hybrid individuals and are therefore of minimal concern. Wells Gulch has the highest number of hybrid individuals with over half the individuals displaying > 10% signal from *S. parviflorus.* The data unexpectedly show nine pure populations of *S. glaucus* and three populations with very low introgression in all but one individual. Microsatellite data reveal additional information surrounding the genetic relationship of *S. parviflorus* and *S. cloveriae.*

STRUCTURE shows that the North of Loma *S. parviflorus* population has extensive introgression from *S. glaucus,* indicating that gene flow is occurring in both directions between *S. parviflorus* populations located in close proximity to *S. glaucus*.

The concern with hybridization is the dilution of the *S. glaucus* genome. If an *S. parviflorus* individual grows within an S. *glaucus* population it could lead to increased hybridization within that population and could be a potential risk for hybridizing with neighboring populations. Chloroplast data for the *trnF(GGA)-trnL-5(UAA)f* and *trnC-rpo-B* regions was used to determine if this was the case in any of the 62 *S. glaucus* individuals sampled. Chloroplast DNA analysis indicated that one *S. parviflorus* haplotype is located within an S. *glaucus* population. Individual SSR6 has a chloroplast haplotype identical to *S. parviflorus* population that was sampled in New Mexico. An *S. parviflorus* microsatellite signal of 87% *S. parviflorus* suggests that SSR6 is either a pure *S. parviflorus* individual or a direct descendant of pure *S. parviflorus*. The data indicate all but one hybrid individual was produced by pollination, but the sampling was limited to 2-3 hybrid individuals per population. The discovery of one *S. parviflorus* individual located in an S. *glaucus* population suggests there is seed or plant movement between populations and additional analyses should be done on all hybrids, especially in the South Shale Ridge populations.

Genetic Relationships of *S. parviflorus* and *S. cloveriae*

There has also been speculation as to whether *S. cloveriae* is a separate species from *S. parviflorus,* or if it is simply a New Mexico variety of *S. parviflorus* (USDA 2012; NatureServe 2012). Although the STRUCTURE, PCoA and chloroplast data indicates that it is not diverged enough to be a separate species, the microsatellite

diversity statistics show that based on genetic differentiation (F_{ST}), *S. cloveriae* is a distinct species. There was only one population of *S. cloveriae* collected and this data indicates that in order to determine species assignment, *S. cloveriae* should be more widely sampled. Group assignment from microsatellite STRUCTURE, PCoA and F_{ST} along with haplotype analyses on the chloroplast regions *trnF(GGA)-trnL-5(UAA)f* and *trnC-rpo-B* would indicate a more in depth investigation between *S. parviflorus* and *S. cloveriae* is warranted.

Sclerocactus Diversity and Structure

STRUCTURE analysis indicates that geographical location is the primary factor driving divergent evolutionary process in *S. glaucus*. *Sclerocactus glaucus* populations have been found in the Gunnison River and eastern Grand Valley drainages and the Colorado River drainage. The populations in the Gunnison River and the eastern Grand Valley drainages form the south *S. glaucus* group, and populations located in the Colorado River drainages form the north *S. glaucus* group. Although there is gene flow between these groups there is not a homogeneous mixing of the two groups. There is higher gene flow from north to south populations and hybridization with *S. parviflorus* is more prominent in the southern populations. This is an indication that perhaps the north populations are more isolated due to the topography of the area.

Genetic distance (F_{ST}) results support the configuration of a north and south *S. glaucus* group and an *S. parviflrous* group. However, based on average F_{ST} values (Table 5), *S. glaucus, S. parviflorus* and *S. cloveriae* are all distinct and separate species. STRUCTURE and PCoA data suggest that *S. glaucus* and *S. parviflorus* are distinct and separate species and should be treated as such. The north and south populations have

genetic distance (F_{ST}) of 0.11 suggesting that these areas hold two divergent *S. glaucus* clusters. The chloroplast genome data did not reveal any distinction between north and south *S. glaucus* populations. This suggests that although the north and south clusters may be diverging, they have not been isolated enough to consider them two distinct species. However, the chloroplast genome is highly conserved and the expectation is that between closely related taxa there would be minimal variation. Therefore it is not unusual that there is no distinction between the north and south *S. glaucus* groups in the chloroplast data. With rapidly evolving or recently diverged species microsatellites are a better indicator for speciation, while chloroplasts can give insight as to direction of hybridization. However, these two unique groups of *S. glaucus* are found in different river drainage systems and therefore may be geographically isolated and could continue to diverge over time.

Sclerocactus glaucus is experiencing the threat of extinction due to not only habitat loss due to human activities, but may also be hybridizing with a close relative that is found nearby, *S. parviflorus.* Land managers need additional information about diversity and hybridization among *S. glaucus* populations in order to preserve the species and maintain the diversity within and among populations.

Microsatellite analyses determined that *S. glaucus* and *S. parviflorus* are distinct and separate species and attempts should be made to minimize hybridization facilitated by human movement. Additionally, two unique groups of *S. glaucus* emerged from the populations found in two different river drainages. The data indicate that these two groups are distinct enough from each other that they should be considered distinct and separate evolutionary units and should be managed as such.

Photographic evidence cross-referenced with genetic data shows that intermediate morphologies do not necessarily reflect the genome of a hybrid individual. In fact, morphological characters, such as hooked or non-hooked spines, are not good indicators for determining species of *S. glaucus* or *S. parviflorus*. Populations such as Wells Gulch, a population with more than 50% hybrid individuals, are not indicative of the genetic structure within *S. glaucus* typical populations. There are a few populations that could present a threat of increased hybridization as many individuals in each population have marginal *S. parviflorus* signal.

The misidentification of Grand Junction Airport and Stage Coach Trail populations indicates that taxonomic identification of *S. glaucus* should not be based on morphology alone. From the populations sampled *S. parviflorus* populations are not seen east of Grand Junction, which indicate there may be geographical distinction as to where the two species thrive. The data indicates that only populations of *S. glaucus* have been found in the Gunnison River drainage and eastern Grand Valley drainages, and in the Colorado River drainage. Therefore *Sclerocactus* populations found in the eastern Grand Valley and Gunnison River drainage systems should be managed separately from populations in Colorado River drainage as north or south *S. glaucus* habitat. Management should avoid moving individuals or populations between these two areas in order to preserve these unique evolutionary units as well as the diversity within the species.

Populations of *S. glaucus* with high diversity levels and minimal *S. parviflorus* genetic introgression should be given conservation priority (Table 9). Analyses of *S. glaucus* have identified the populations of *S. glaucus* that have high diversity, low inbreeding and low levels of hybridization. Interestingly, 16 of the 28 *S. glaucus*

populations had little to no *S. parviflorus* introgression. These populations along with diversity measures (Table 9) should give land managers and conservation biologists the information they need to make decisions surrounding the preservation of this species. As management resources allow, all *S. glaucus* populations are important and should be preserved, monitored, maintained and managed.

The *S. glaucus* population Wells Gulch has a relatively high degree of introgression from *S. parviflorus.* This population should be isolated from other *S. glaucus* populations to minimize the possibility of introgression into adjacent populations. Additional analyses should be conducted to determine if there are *S. parviflorus* individuals among this population. Individuals with high *S. parviflorus* genetic signal should be removed to reduce the possibility of further contribution of *S. parviflorus* genetic material to *S. glaucus* populations.

The chloroplast DNA data reveled that one individual from the north *S. glaucus* population, South Shale Ridge, has an *S. parviflorus* chloroplast haplotype. This individual should be located and removed from this population to curtail *S. parviflorus* hybridizing with *S. glaucus* within and with surrounding populations. Moreover, a more substantial cpDNA analysis on this population is required to ensure there are not other *S. parviflorus* individuals within South Shale Ridge or the populations adjacent to it.

Conservationists are concerned about the future of *Sclerocactus glaucus* because it is a rare endemic Colorado species. There are many threats to *S. glaucus* including habitat disturbance from oil and gas exploration, urbanization, open range cattle grazing and recreational land use by humans. Additionally, due to the low numbers found in the wild, the genetic integrity of the species was in question. Observations from field

biologists observed *Sclerocactus glaucus* populations with individuals that appeared to be either hybrids or individuals of a closely related species, *S. parviflorus.* Using genetic tools it was established that *S. glaucus* populations remain diverse and mostly untainted by hybridization. Land managers and conservationists now have the genetic information to move forward with preserving populations of *S. glaucus.*

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