Phylogeny and Population Genetics of the Endangered Dwarf Bear-poppy, Arctomecon humilis Coville (Papaveraceae) Using Microsatellite Markers

Joshua Simpson
Graduate Center, City University of New York

by

Joshua M. Simpson

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This manuscript has been read and accepted by the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

Date
Chair of the Examining Committee
Dr. Dwight Kincaid
Lehman College, CUNY

Date
Executive Officer
Dr. Laurel Eckhardt

Date
Dr. Damon Little
The New York Botanical Garden

Date
Dr. Renee Van Buren
Utah Valley University

Date
Dr. Robert Naczi
The New York Botanical Garden

Date
Dr. Joseph Rachlin
Lehman College, CUNY

THE CITY UNIVERSITY OF NEW YORK
Abstract


by

Joshua M. Simpson

Advisor: Dr. Dwight Kincaid

The genus *Arctomecon* (Papaveraceae) is comprised of three narrowly endemic rare species that are largely restricted to gypsum soils of the eastern Mojave Desert. The small, remaining populations of these species have become increasingly isolated by urban development and habitat fragmentation. *Arctomecon humilis* is federally listed as endangered due to its limited distribution within a ~15 km radius of an actively expanding city. Organizations involved with land management and conservation have called for greater insight into the genetic variation and population structure of the remaining subpopulations as they make important decisions regarding where to focus their efforts and resources.

The goal of this study was to provide answers to some of the remaining research gaps involving *Arctomecon* species particularly conservation genetics by developing microsatellite markers and comparing community dynamics. First, a phylogenetic study using six gene regions (*nrITS*, cpDNA (*matK, rbcL, trnH-psbA, rpl32-trnL, ndh-rpl32*)) was conducted for members of the genus and three outgroup species. Thorough sampling throughout the geographic range of *Arctomecon* was conducted in order to obtain a complete representation of the genetic variability present across multiple populations. A total of 1176 plants were sampled from 35 locations with DNA extraction being performed on 949 of those, to be included in different stages of research. Outgroup taxa included a member of the sister genus *Argemone*, a
Meconopsis species from within the subfamily, and an Eschscholzia from a separate subfamily. It was hypothesized that this increased sampling and number of gene regions would provide a more robust species tree, as compared to previous studies. Additionally, I hypothesized that new genetic markers could identify isolated populations that would be more informative to conservation management. The phylogenetic analysis did result in a well-supported species tree in addition to exhibiting broad structure among populations within each species. Notably, the population sampled in the Grand Canyon is genetically and morphologically divergent from all the other populations of A. californica that were sampled.

Polymorphic microsatellite markers revealed the micro-evolutionary structure from within and between populations of A. humilis. This was the first time that genetic markers of this type have been developed for any Arctomecon species. Sixteen markers with 2 to 31 alleles (mean=12) per marker were used to determine the level of variation and admixture among 341 individual plants from thirteen sampling localities. The number of individuals per locality ranged from 26 at Price Hills to 49 at Boomer Hill. Each marker was tested for amplification and variability within the sister species A. californica and A. merriamii where cross-amplification occurred with less success and fewer alleles than in A. humilis.

Population genetic analyses identified localities with greater amounts of admixture, as well as those more isolated and at risk of inbreeding depression. Through Bayesian analysis and genetic cluster assignment the overall trend suggests that populations are becoming more isolated. Analysis of Molecular Variance found 30% of the genetic variability between populations, and the F_{ST} analogues indicated substantial genetic differentiation (G’_{ST}=0.427). A concern among land managers and conservation organizations concerned the effectiveness of the reserve system. An analysis of the allele frequencies located within the protected areas does indicate that the reserve system is effectively capturing genetic diversity. However, allele
frequency data also suggest that a small number of new annual recruits represent only a subset of potential alleles. Due to the small effective population sizes and the already rare habitat supporting *Arctomecon humilis* the conservation efforts should continue to monitor and protect this unique species in all locations.
Preface

This dissertation contains an overall abstract, four chapters and three appendices. Each chapter will be adapted to a manuscript format. Portions of the background information and methods may be repeated where it is applicable to the research being described. Tables and figures appear at the end of each chapter, and a bibliography that includes all references cited throughout the dissertation is located after the fifth chapter.
Acknowledgements

First off I would like to thank my advisor Dr. Dwight Kincaid not only for providing me the opportunity to conduct the research described in this dissertation; but for guidance through the CUNY program in general. I am grateful for all the support and guidance he provided over the years and his willingness to openly discuss any topic at hand. I would also like to thank the members of my committee: Dr. Damon P. Little for his guidance, support, and excellent understanding of molecular evolution and bioinformatics; Dr. Robert Naczi for his passionate approach, positive attitude and flexibility, as he is constantly involved in numerous projects; Dr. Joseph Rachlin for his straight forward critiques, and willingness to offer help when needed; and Dr. Renee Van Buren for introducing me to the wonderful focal species of this research, she helped instill in me a love of conservation and an active drive toward environmental issues.

I must also acknowledge the essential support and ideal laboratory setting of the New York Botanical Garden’s Pfizer Plant Research Laboratory, and the numerous individuals that I have been able to interact and work with there. The lab facilities themselves are state of the art, with lab technicians always willing to lend a helping hand.

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Micronesian plant collections, georeferencing and mapping projects. Dr. Wayt Thomas and Dr.
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Reginato. They are constantly exploring and expanding their knowledge base, excelling at
everything they start and taking it further than anyone else.

Many people were integral in locating and acquiring the samples necessary to conduct
this research and, in particular, I would like to thank the late Dr. Kimball Harper for taking me
on plant collecting excursions in which his vast encyclopedic knowledge of the plants, geology,
and history of an area always kept hikes interesting; he was a true naturalist of the Intermountain
West and Great Basin region. Dr. Susan Meyer and her expert knowledge of the genus, as well as
the best places to find populations proved invaluable. I also appreciate the help I received from
Wendy Hodgson and Art Phillips in getting me to the Grand Canyon population. I must acknowledge the funding and help provided by the U.S. Fish and Wildlife Service, without which this work could not have been completed. The National Park Service in Death Valley, Ash Meadows, and Calico Basic helped me locate small populations that I would never have found on my own. The Nature Conservancy has played one of the most prominent roles in garnering support and protection for these species, and their help was essential around St. George, UT and Las Vegas, NV where they have purchased numerous reserves. Employees of TNC offered advice, and direct help accessing reserves, GIS data, and locating plants. Bureau of Land Management employees, and Las Vegas Airport staff also provided access to populations. Many individuals also helped get collecting permits in place, which was essential to this project.

My wife Alicia and her organizational skills, never-ending support, and partnership must also be acknowledged as my sincere appreciation goes out to her; and our children, Tatjana, Seppo and Terai, who helped keep my life balanced and provided needed distractions and escapes. They kept me motivated and excited along the way. I am also indebted to many friends and family members outside of the scientific community, without which I would not have been able to complete this dissertation. My father-in-law was always willing to provide me with a vehicle for field work, which was absolutely invaluable and allowed me to access areas that I would not have been able to get to in a timely manner otherwise. Robin and Kenny McCorristin for their encouragement and help keeping my kids entertained. This also includes the NY longboarding community, Cameron Van Tassel, Ben Peters, Slade Combs and the Morningside Heights YM, as well as the crew down at the Hudson Beach Rings, Ira, Heath, and others.

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<tr>
<th>Population</th>
<th>Sample Size</th>
<th>Observed Heterozygosity</th>
<th>Expected Heterozygosity</th>
<th>Fstatistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population A</td>
<td>100</td>
<td>0.60</td>
<td>0.55</td>
<td>0.05</td>
</tr>
<tr>
<td>Population B</td>
<td>150</td>
<td>0.70</td>
<td>0.65</td>
<td>0.02</td>
</tr>
<tr>
<td>Population C</td>
<td>200</td>
<td>0.80</td>
<td>0.75</td>
<td>0.01</td>
</tr>
</tbody>
</table>

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Chapter 1: Introduction and Background to the Genus *Arctomecon*

Introduction

The genus *Arctomecon* Torrey & Frémont is a member of the Papaveraceae family containing three species commonly known as the Desert bear-poppies: *A. californica* Torrey & Frémont, *A. humilis* Coville and *A. merriamii* Coville (Nelson and Welsh 1993). Both the common name and *Arctomecon* (Greek arktos, of bear; and mekon, a poppy) allude to the shared morphology of their leaf structure; pubescent leaves with the distal margins lobbed and tipped with trichomes resembling bear paws.

All three species are rare endemics of the northern Mojave Desert ecoregion in the southwestern United States. The extent of their geographic distribution and limited range is linked to soil characteristics, primarily the presence of gypsum containing substrates. *Arctomecon humilis* has the narrowest geographic range of the three species, found only within a 15 km radius of St. George, UT (Figure 1-1). Soils from all population sites that have been tested and are known to support *A. humilis* contain 27-51% gypsum (Nelson and Harper 1991). These populations are restricted to the upper layers of the Moenkopi formation, most often associated with the Shnabkaib Member, but also on the Upper and Middle Red members (USFWS 1985). It is the only species in the genus that is federally listed under the Endangered Species Act (USFWS 1979), though it has been recommended that all be designated as endangered (Nelson and Welsh 1993).

Both *A. californica* and *A. merriamii* inhabit gypsum rich soils, but they are less soil-restricted than *A. humilis* (Meyer 1986, Drohan and Merkler 2009), and consequently have wider distributions. The White bear-poppy, *A. merriamii* has the widest range of the three species, with locations ranging from its westernmost point in the Death Valley region of California to its
easternmost point in the Las Vegas Valley. However, many of the populations have fewer than 100 individuals according to recent surveys (The Nature Conservancy (TNC) 2007a, and personal observation). *Arctomecon californica* is found within the Las Vegas city limits, east to the Lake Mead National Recreational Area (LMNRA) in Nevada and adjacent Arizona, and with at least three small populations further south in the lower Grand Canyon National Park and contiguous Hualapai Reservation. These populations also vary greatly in size with some populations having thousands of individuals and others with fewer than one hundred (TNC 2007a, and personal observation). The habitat characteristics, including chemical and physical soil parameters as well as biotic associates have been described for all three species at multiple locations (Nelson and Harper 1991, Sheldon 1994, Mistretta et al. 1996). The gypsophile habit of *A. californica* has recently been challenged, and the facultative term gypsovag has been proposed for this species as it can tolerate high gypsum content, but does not require it (Drohan and Merkler 2009). Not surprisingly, gypsum content in the soil is merely a contributing factor at many sites, while a combination of soil characteristics actually determines the presence of these species (Drohan and Merkler 2009).

All three *Arctomecon* species are short-lived perennial herbs (average lifespan ~5 years) with evergreen leaves forming dense rosettes from taproots. They share an ample number of traits that easily show their apparent affinity to each other, while morphological differences among them demonstrate probable taxonomic delineation (Figure 1-2, Table 1) (Welsh et al. 1987, Intermountain Flora).

*Phylogeny of the New World Poppies*

The systematic relationships among North American genera of desert poppies (*Arctomecon, Argemone, Romneya, Canbya, Platystemon, Hesperomecon and Meconella*) have
proven to be problematic due to extremely diverse and unique morphology of each genus. Studies attempting to construct a phylogeny based on morphological characters within genera combined with cpDNA found that the Old World clade (including the New World *Papaver californicum* and *Papaver heterophylla*) was clearly monophyletic, identifying 17 synapomorphies. However, among the North American desert group there were far more molecular autapomorphies within each taxon as compared to morphological synapomorphies. This result led to poorly supported basal branches in the New World clade (Kadereit *et al.* 1994, Schwarzbach and Kadereit 1995).

A follow-up study by the same authors, primarily attempting to resolve the monophyly of the genus *Argemone*, and secondarily to determine relationships within the New World clade, included 24 species of *Argemone* (47 individual plant specimens) and seven other genera (one individual each) (Schwarzbach and Kadereit 1999). It verified monophyly for *Argemone* as well as established *Arctomecon* as its sister group. However the relative position of the other outgroups to one another was unclear, possibly due to small sampling regime. This result could be due to a genetic marker with poor resolution among these groups, though the researchers do not believe that to be the case. The authors explain the lack of resolution being due to rapid diversification and ecological adaptation in an arid environment (Schwarzbach and Kadereit 1995, Schwarzbach and Kadereit 1999). In order to highlight the possibility of a rapid diversification leading to highly diversified morphology, the authors use *Arctomecon*, as an example of a small morphologically distinct genus. *Arctomecon* differs from closely related genera by having cuneate leaves arranged in rosettes and densely covered by long hairs. They possess a non-reticulated seed coat surface (while *Argemone* and *Romneya* both have fine reticulation) as well as the presence of an aril, which the others lack. Finally, species of
Arctomecon have unique alkaloid chemistry and the genus contains the only known gypsum obligates in the Papaveraceae (Nelson and Welsh 1993, Schwarzbach and Kadereit 1999).

While Arctomecon is distinct when compared to the two most closely related genera, the three Arctomecon species are also very distinct from one another. For instance, although A. humilis and A. californica were identical in their restriction fragment length polymorphism (RFLP) site pattern in Schwarzbach and Kadereit (1995), these two species are geographically isolated from each other and have many morphological differences (refer to Figure 1-2, Table 1).

There has been no agreement on which species within Arctomecon is sister to the rest of the genus. Schwarzbach and Kadereit (1999), state that A. humilis is morphologically the most derived and A. californica is nested within the other two species, citing unpublished data. Other studies found that A. californica and A. merriamii were more similar and likely derived while A. humilis was ancestral according to alkaloid chemistry and the type of trichomes present (Raynie et al. 1991, Mistretta et al. 1996, respectively). By resolving the phylogeny of this genus a clearer picture of the New World clade will be obtained. For example, A. humilis has been included in phylogenetic studies as the only representative of the genus. If this is the most derived species it may actually be more beneficial to select the most ancestral species, and reduce the amount of potential autapomorphies.

Current Threats

All three Arctomecon species are threatened due to the highly specific habitat they require, combined with the speed at which urbanization is occurring around many of their remaining populations. The substrates supporting Arctomecon populations are sparsely vegetated, and are often associated with biological soil crusts typical of arid environments. These biotic crusts, as well as abiotic crusts keep the soils intact and lessen erosion. These delicate
habitats are easily destroyed by physical disturbance, which can be as simple as foot traffic, but
are largely irreparable with more extensive damage caused by off-road vehicles, mining, and
construction. While Nelson and Welsh (1993) recommended all three species to be covered
under the endangered species act, *A. humilis* is the only one currently listed as a federally
endangered species (1979). *Arctomecon californica* is listed as critically endangered by the state
of Nevada and imperiled in Arizona. *Arctomecon merriamii* is listed as vulnerable in Nevada,
and imperiled and threatened in California (TNC 2007a). The criteria largely correspond to the
IUCN’s Vulnerable category, due to their rapid population decline (Van Buren and Harper 1996,

Habitat degradation and fragmentation are harmful to all species, but can be of serious
concern to rare endemic species with narrow ranges because these threats lead to stochastic
vulnerability of small populations. Urban development and environmental changes are occurring
at unprecedented rates throughout the *Arctomecon* habitat. From 1990 to 1998, Nevada, led all
U.S. state growth statistics, with the majority of the population settling around Las Vegas
(Hickerson and Wolf 1998). Clark County, Nevada, increased its population size by 63% within a
7-year period (1993-2000) to 1.45 million when it had been predicted to take almost 30 years to
attain that population size (TNC 2007a). The current population of Clark County stands at just
over 2 million (U.S. Census Bureau 2012). Additionally, Washington County, Utah, increased its
population size by 86.1% between 1990 and 2000, making it the 12th fastest growing county in the
country during that period of time (U.S. Census Bureau 2001). Washington County continued to
grow to a current population just above 140,000 (U.S. Census Bureau 2012). While this total may
not seem high by some standards, it is important to note that the entire species distribution of *A.
humilis* is within a 15 km radius of St. George, UT, the most populous city in Washington County.
This rapidly developing and expanding population will inevitably continue to clear and manipulate
land, resulting in by-products of environmental degradation in the form of soil erosion, habitat loss, altered land-use patterns, and physical and chemical pollution, among other detrimental effects.

According to long-term demography, the seed bank has been cited as an essential conservation priority (Van Buren and Harper 1996, Allphin et al. 1998, Hickerson and Wolf 1998). The seed bank is important from a demographic and ecological point-of-view, since all three species exhibit large year-to-year fluctuations in population density and are known to exhibit mass seedling emergence in favorable years (Harper and Van Buren 2004, Meyer personal communication). This is a typical pulse-reserve strategy observed in desert species of extremely arid environments, where bursts of activity follow heavy rains (Reynolds et al. 2004). Seeds of *Arctomecon* possess seed coat dormancy with a slow progressive loss of dormancy over time, an adaptation to the drought cycles of the Mojave (Drohan and Merkler 2009, personal communication Meyer). In years of abundant rainfall, non-dormant seeds will germinate en masse and a small number of seedlings will be established (Harper and Van Buren 2004). Established populations have been known to experience gradual loss of individuals over time, with multiple drought years leading to lack of new seedling recruits, resulting in population dormancy. In these cases, the dormant seed bank avoids periods of extended drought, and the population will be re-established when conditions become favorable again (TNC 2007a).

Actively expanding urban areas represent an ideal case to study ecological and social processes and their effect on environmental health. An improved understanding of the impact that fragmentation has on populations of rare endemic species, their ecological interactions and their viability as they experience rapid development should then influence policy implementation by city and state government agencies. As environmental disturbances, such as habitat degradation and fragmentation, increased competition by invasive species, and off-road vehicle (ORV) traffic
continue to worsen, it is likely that the viability of plant populations in this area will be impaired (USFWS 2006). Recent studies of *A. humilis* showed that as fragmentation proceeded or population density was reduced, the percent pollination and seed number per fruit were significantly depressed (Harper and Van Buren 2004). This was likely due to the fact that pollinators either had less success finding the few existing plants and/or their behavior changes as a consequence of increased interplant distances (Oostermeijer *et al.* 2003, Kearns *et al.* 1998). Reduced pollinator success will likely lead to decreased genetic diversity within the disturbed populations. Decreased genetic diversity can in turn lead to a reduced ability of a species to respond to environmental changes and pressures, leading to increased risks from inbreeding and eventually the risk of extinction.

*Current Status*

While each of the *Arctomecon* species has some populations or sub-populations in protected areas, the continued loss of habitat remains of concern (Table 1-2). The majority of *A. humilis* populations are currently protected in reserves or areas closed off to ORV use. This is the result of a combined effort between the Bureau of Land Management (BLM), the U.S. Fish and Wildlife Service (USFWS), Utah’s School and Institutional Trust Lands Administration (SITLA) and TNC. In 2011 they completed their goal of establishing an 800-acre reserve system (Havnes 2011). Small populations that are not in protected areas are likely to be lost to development. Some populations of *A. merriamii* are in Death Valley National Monument, CA, and protected by the National Park Service. The largest reserve, and the largest populations for this species exist in the Desert National Wildlife Refuge, NV, as well as the adjacent Nellis Air Force Base. The other populations of this species tend to be few and far between. Protected sites for *A. californica* populations are found on Bureau of Land Management property in the
Lake Mead Recreational Area, Valley of Fire State Park, NV, and Whitney Pocket in the Gold Butte area east of Lake Mead. Additionally, TNC has one reserve within the Las Vegas City limits and there is one population on the property of the Las Vegas Airport, which is essentially protected due to limited access of the area. Fortunately TNC is currently planning further land acquisition as additional habitat reserves.

Long term demographic plots have been consistently monitored for two of the three species (*A. humilis* and *A. californica*) and have provided valuable information for the population biology, and sampling design of studies involving the bear-poppies (Harper and Van Buren 2004, Meyer in preparation, respectively). Population genetics without demographics in conservation has often led to less than useful recommendations (Oostermeijer *et al.* 2003, DeSalle and Amato 2004). The need for appropriate population level studies using molecular data to determine the current state of gene flow within and among the different populations of each of these species still exists. While there have been molecular studies conducted in the past involving some of the populations (Van Buren and Harper 1996, Allphin *et al.* 1998, Hickerson and Wolf 1998), there is a need for finer scale analysis to inform population level questions. Recovery plans for these species will be greatly improved by a better understanding of genetic diversity and gene flow among the remaining populations.

*Previous Studies*

There have been three studies involving molecular data at the population level for the *Arctomecon* species. The first measured variation among populations of *A. humilis*, as well as between the three *Arctomecon* species, an *Argemone*, *Romneya* and *Papaver* sample using Randomly Amplified Polymorphic DNA (RAPDs) (Van Buren and Harper 1996). This study grouped all individuals of each population into a single sample. This showed very little variation
among populations of *A. humilis* or *A. californica* (95% and 93% similarity, respectively), while *A. merriamii* showed 68% similarity between populations. While this study showed variation between species (15-23% similarity at the chosen markers), it was unable to offer insights into genetic variation within populations, as population level samples were combined.

The other two molecular studies used allozymes to measure differences within and among populations of *A. humilis* and *A. californica* (Allphin et al. 1998, Hickerson and Wolf 1998, respectively). Hickerson and Wolf, sampled 16 populations with four populations in fragmented habitats and twelve populations in unfragmented habitats, and found high variation between the populations, although lower variation in fragmented landscapes (1998). Allphin *et al.* (1998) sampled six of eleven populations and collected specimens from three separate age classes as defined by size and presence of flowering stalks. They found greater variation among populations as compared to the RAPDs study (Van Buren and Harper 1996). Interestingly, among the allozyme loci that were analyzed, it was found that the two westernmost populations of *A. humilis* shared additional loci with the nearest *A. californica* population that were not found in other populations of *A. humilis* (Allphin *et al.* 1998). Considering how morphologically different these species are, and how robust the species level determination is, this finding could have been due to historic gene flow or common ancestry (Allphin *et al.* 1998). However, these markers may not provide sufficient data to interpret contemporary gene flow between populations and species (Harper, personal communication 1998). Although variation between the age-classes was seen with the intermediate stage being different from seedlings and older plants, the authors note age-class sampling was limited and did not allow for accurate statistical analyses at this level (Allphin *et al.* 1998).
Landscape Genetics

Genetic data have become increasingly beneficial in conservation biology because it allows new insights and novel approaches. Particularly, by combining this fine scale genetic data with landscape characteristics it provides a greater understanding of the underlying processes and patterns that structure a population (Manel et al. 2003). This fine-scale phylogeographic approach has been termed ‘landscape genetics’ and is aimed at discerning genetic discontinuities and correlating them with environmental features or barriers (Manel et al. 2003). *Arctomecon* provides an ideal example for testing such hypotheses regarding the effects of natural and anthropogenic habitat fragmentation on patterns of genetic variation, and allows the opportunity to compare among populations with and without habitat reserves. By employing landscape genetic methods, variation in populations and in individuals within populations can be used to glean information on ecology, evolution, and conservation of the target species.

Conservation strategies are of particular interest throughout the region where *Arctomecon* can be found because of the unique geology and floral diversity in the Mojave Desert ecoregion, near the Great Basin. Washington County, UT, has the highest number of endemic plant species in Utah, and contains some of the most unique landscapes and habitat (Welsh et al. 1987). Additionally, Clark County has the second highest number of rare and endemic plant species in Nevada, and many of the endangered species of highest concern (Nevada Natural Heritage Program 2009).

*Arctomecon humilis* is known only from a small area in Washington County, Utah, and is considered to be one of the most endangered plant species in that state due to urban growth (Welsh and Chatterly 1985). Although it was Federally listed as endangered in 1979, and a recovery plan was proposed in 1985, the USFWS did not designate any protected areas or fund the recovery. A number of dedicated botanists have devoted time and resources to studying this
species, but it was not until the mid-1990’s when some of the populations were under severe threat, that a concerted effort was put forth to protect the remaining populations. Since that time, a number of organizations have become increasingly involved in the protection and preservation of the species, bringing more attention to the endangered plant species of the region. It has since become a flagship species of conservation efforts, particularly in Washington County, but also throughout the state. In 2007, the city of St. George declared that the second Saturday of May would be known as Dwarf Bear-Poppy Day (TNC 2007b). Due to the increased interest in the preservation of this species, the USFWS and TNC expressed an interest in knowing the dynamics of the population genetics of this species. They wanted to know the fine scale genetic variation of these populations and get a better idea of the population structure within the remaining locations.

Research Objectives

The goal of the current study was to develop microsatellite markers and compare community dynamics and conservation genetics involving *Arctomecon* species. Biologically sound conservation and management decisions about endangered species require knowledge of how the species is structured spatially, the amount of gene flow throughout that distribution, and to what extent its populations interact demographically (Coulon *et al.* 2008). My development of microsatellite markers for the genus *Arctomecon* has helped to elucidate relationships within these species on a number of genetic levels. First, focus was placed on the phylogeny of the genus such as determining which species is sister to the rest of the genus. This helped to untangle some of the confusion between the genera of the New World desert poppy clade. The second goal of this study compared the diversity of microsatellite genotypes between the geographically restricted *A. humilis* with the two more widespread sister species, allowing
greater interpretation of diversity within these populations. The third and major aim of this research was to gain increased resolution of the population biology and conservation genetics of the federally listed *A. humilis*. This was determined through the analysis of genotypic data of the microsatellite alleles. This deeper understanding of distribution and diversity at the interspecific and intraspecific levels will contribute to improved long-term management among these unique species.

A number of hypotheses were tested using molecular data among the *Arctomecon* species in these unique habitats and reserves. One of the primary concerns was to provide a better definition for a “population” within this landscape. Considering that *A. humilis* was likely one large contiguous population that was first subdivided by the erosion of gypsum beds (Van Buren and Harper 1996), and more recently by human disturbance, the manner in which the current sub-populations were sampled could impact the results of these studies. In the past, studies conducted on *A. humilis* have pooled samples based on geographic locations prior to analysis (Van Buren and Harper 1996, Nelson and Harper 1991). This predetermination may bias the results, as some populations are based on management boundaries and not population dynamics like pollinator interactions. For example, Warner Ridge and Beehive Dome are likely contiguous with each other, as are Price Hills and Webb Hill, since they are within fenced enclosures on opposite sides of a relatively recently constructed intersecting road (Van Buren, personal communication). Sampling was conducted at each of the currently named sites, keeping track of their location, but the analyses were run considering all individuals independently regardless of collection location (Manel *et al.* 2007, Corrander *et al.* 2009). This approach is more likely to reveal cryptic population structure and gene flow dynamics (Manel *et al.* 2007, Jombart *et al.* 2008).
Hypotheses

1. The phylogeny and phylogeography of the species within this genus can be clarified using new and additional gene regions. This hypothesis was tested, using six gene regions (*nrITS*, cpDNA (*rbcl*, *matK*, *rpl32-trnl*, *ndh-rpl32*, *trnH-psba*) and assessing each for phylogenetic resolution. The gene regions were selected based on their use in DNA barcoding at the species level. Additionally, sequences were obtained for multiple individuals per population in order to determine whether genetic differentiation exists at the population level using these broad markers. This was done in comparison to prior genus level studies that have been conducted for the New World Papaveraeae. This hypothesis was rejected when the new markers provided a significant amount of variation between these morphologically distinct taxonomic groups.

2. I hypothesized that populations of *Arctomecon humilis*, or genetic clusters, as identified by Bayesian population structure programs would differ from their traditionally defined management units, and that they would form broader population groups. This hypothesis was partially supported as sub-populations grouped together based on their geographic location around the city. However, the analyses showed that plants at ten of the thirteen collection locations were assigned to distinct populations with high probability, with limited admixture between collection sites. This information will be directly useful in establishing evolutionarily significant units for management purposes.

3. I hypothesized that the current reserve design had not effectively captured the genetic diversity of the species. Since this species is identified as a conservation flagship among the desert flora, we wanted to test whether the reserves are set up in a manner that is conserving the species on a long-term trajectory by determining whether reserves are encompassing the
genetic variability found throughout the species range. This hypothesis has been rejected because 1) new reserves have been established since the initiation of my project, and 2) according to allelic diversity of the microsatellite markers the reserves are adequately preserving a wider range of genetic diversity within the species as compared to plants located outside of the reserves. Protected areas were found to be supporting more genetic diversity than the non-protected sites. A similar hypothesis was analyzed among fragmented (small population groups) and unfragmented (large populations) sites, where intact populations were expected to maintain diversity while fragmented populations had lower diversity. The small fragmented sites, or more isolated sites, were found to possess fewer genotypes and have less variation overall as compared to the larger population sites.

4. I tested the hypothesis that the different age-classes (seedlings, juveniles, flowering adults) of *Arctomecon* would show different levels of variation, with seedlings exhibiting the highest amount of diversity. This hypothesis was based on the fact that seedling emergence events were expected to represent the entire genetic gamut due to the ‘pulse-reserve’ mechanism that these species have evolved. The microsatellite markers were able to detect similarities and differences between genetic source categories. However, based on the observed results, the hypothesis was rejected, because there was less variation among the seedlings and juveniles as compared to the adults.

*Phylogeny of the Genus*

A combination of molecular markers was employed to resolve the phylogeny of the three species within the genus. A previous study sequenced the *nrITS* region, but only included a single species (*A. humilis*) in their analysis, in comparison to *Argemone* (Schwarzbach and Kadereit 1999). To extend this information, samples from multiple individuals per population of
each species were sequenced to determine if broad level structure across their geographic
distribution could be detected. In addition to *nrITS*, other typical species barcode regions
(cpDNA *trnH- psbA, ndh-trnL, matK*) were sequenced and concatenated along with the *nrITS*
markers that proved to be most useful at the genus level were the gene regions containing SNPs
(single nucleotide polymorphisms). Outgroups were included for all analyses including a species
from the sister genus *Argemone*, a *Meconopsis* species from within the subfamily, and an
*Eschscholzia* from a separate subfamily. Obviously, better resolution was expected by
incorporating samples from more individuals of each species, and adding more gene loci to the
analysis than previous studies had done.

*Population Genetics*

The previous studies involving molecular data of *Arctomecon* species mentioned above,
provide good baseline genetic information (Van Buren and Harper 1996, Allphin *et al.* 1998,
Hickerson and Wolf 1998). However, a more in-depth population study using sensitive
molecular techniques to assess genetic variability at loci that exhibit increased polymorphism at
the population level is necessary (Allphin *et al.* 1998). The microsatellite markers that have been
developed for this project have provided data further resolving infraspecific gene flow, as well as
interspecific allele frequencies. Microsatellites are co-dominant nuclear DNA markers that
consist of short, repeated nucleotide sequences (between 1 and 6 base pair motifs repeated)
(DeSalle and Amato 2004). Microsatellites have proven to be useful in analyzing short or fine-
scale population processes, such as contemporary estimates of migration. They can be used to
estimate relatedness within and between populations through individual identification and to
resolve parentage of interacting individuals (Sunnucks 2000, Selkoe and Toonen 2006). Studies
that have directly compared microsatellites, allozymes, and RAPDs have found that microsatellite loci are more useful in detecting variation for studying the genetic population structure of a species (Chen et al. 2007, Sun et al. 1998, Hughes and Queller 1993).

Microsatellites have facilitated a direct approach to measuring gene flow (Ouborg et al. 1999). They can be used to detect recent population level changes (Selkoe and Toonen 2006, Gebremedhin et al. 2009), which would be most valuable in this fragmented landscape of conservation concern. Microsatellites have also proven to be advantageous over allozymes, particularly for conservation studies, because DNA can be extracted from old samples (i.e. herbarium specimens). This DNA is more convenient for collection, sequencing, and storage of samples, and the markers are more variable, and PCR assayable (Sunnucks 2000, Selkoe and Toonen 2006). Microsatellites are also advantageous over RAPDs in that they provide allele frequencies rather than just the estimation of genotype (co-dominant vs. dominant marker), and give much better resolution at within population variability (Ouborg et al. 1999).

**Research Methods**

The conservation genetics and population biology research was divided into three major areas, each involving a different taxonomic unit.

- First, a phylogenetic determination of the species’ relationships and genetic distance within *Arctomecon* was conducted, with other Papaveraceae genera as outgroups.
- Second was an assessment of genetic variation across all of the *A. humilis* populations based on six gene regions and microsatellite marker amplification, with a comparison to the level genetic variability across *A. californica* and *A. merriamii* populations.
Third was the infraspecific determination of genetic structure at the population level throughout the geographic range of *A. humilis* using allele frequency data acquired from microsatellite loci.

Thorough sampling throughout the geographic range of all three species provided a complete representation of the genetic variability of the genus. Additionally, ecological transects were used for a quantitative plant ecological inventory at most *A. humilis* sites. To ensure that sampling points were not biased, they were selected along transects using a random number generator.

In order to develop microsatellite markers all molecular methods were conducted under the direction of Dr. Damon Little in the Pfizer Plant Research Laboratory at The New York Botanical Garden (NYBG). The original goal was to identify a minimum of eight polymorphic, variable microsatellite loci that would consistently amplify across all three species. However, this goal was modified in response to a lack of marker amplification or highly reduced allele variability across the genus. An alternative goal of obtaining a minimum of 12 polymorphic loci that were variable within the focal species, *A. humilis* was established.

*Sampling Protocol and DNA Extractions*

After preliminary scouting visits were made to numerous populations, a sampling protocol for collection of leaf tissue was designed. One hundred meter transects were set up to obtain a random subset of 30 individuals at each population. DNA extractions were acquired from the leaf tissue samples shortly after returning to NYBG from the field. The initial DNA samples were used as the source for creating the genomic library that was subsequently used to develop the microsatellite markers. More extensive sampling occurred during the next three field seasons. A subset of the additional samples was then used in preliminary testing of all
microsatellite markers and later for the complete genotyping and analysis of genetic structure among members of the genus, and within *A. humilis*.

*Molecular Methods*

Although the three species of *Arctomecon* are morphologically distinct, a robust determination of the phylogenetic relationships is lacking. As part of the present study, we provide a more comprehensive phylogenetic treatment of the genus by using multiple chloroplast regions, and one nuclear locus. One purpose of including multiple individuals per population was to verify the divergence of the Grand Canyon population of *A. californica*, as it had been noted as having a unique habitat, and being morphologically distinct. Gene regions were run independently and each tree was constructed using maximum parsimony in TNT (Goloboff *et al.* 2008). The six regions were then concatenated and run through TNT under maximum parsimony, and were additionally uploaded to *BEAST* (pronounced ‘Star Beast’, Heled and Drummond 2010) to obtain a species tree estimation using a full Bayesian framework.

Microsatellite markers were isolated from a genomic library, constructed from leaf tissue extracted DNA of *Artcomecon humilis* following established protocol (Edwards *et al.* 1996, Little *in prep*). Additionally, two other microsatellite enrichment techniques (membrane-enrichment and magnetic bead-enrichment) were tested for levels of efficiency. While these methods proved to be fairly effective in developing molecular markers, one further method, the 454-sequencing high throughput technology was also used in order to determine the best method of microsatellite primer development for species of conservation concern (Ellegren 2008).

As the microsatellite markers were discovered, they were sequenced and screened for variability among at least 24 samples representing individuals from within and between the *Arctomecon* populations and species. The goal was to obtain microsatellite loci that were
successfully amplifying across all three species. Additionally, monomorphic or inconsistent loci were not pursued further. Once suitable polymorphic microsatellite loci were identified (following criteria in Selkoe and Toonen 2006) the remaining samples were run through the same optimized procedure and sequenced for population genetic analysis. The number of alleles per microsatellite locus, observed \((H_o)\) and expected heterozygosity \((H_e)\), conformance to Hardy-Weinberg equilibrium (HWE) expectations, and population genotypic disequilibrium using pairwise tests were then assessed using GenAlEx software (Peakall and Smouse 2006). Further analysis of the genotypic frequencies and population structure were then conducted.

**Data Analysis**

Once the final set of microsatellite primers was developed and polymorphic loci had been identified, the genotyping of multiple individuals (4-30) per population site was conducted and allele frequencies were determined. The original goal was to genotype 30 individuals per collection site, however fewer than 30 individuals were located at a number of sites. At the population (collection site) and species level analysis classical summary statistics were obtained for each marker, followed by Bayesian approaches (Excoffier and Heckel 2006), in order to understand genetic diversity at the different levels of biological organization and spatial distribution (Manel et al. 2003, Manel et al. 2007). These respective associations were then analyzed to infer true population groups based on genetic clustering algorithms as well as potential barriers to gene flow. Population dynamics were assessed through Analysis of Molecular Variance using GenAlEx (Peakall and Smouse 2006). Genetic variation was analyzed for population groups, including: allele frequencies, percent polymorphic loci \((P)\), mean number of alleles per locus \((A)\), mean observed heterozygosity \((H_o)\), and the expected heterozygosity under Hardy-Weinberg expectations \((H_e)\). Additionally, Wright’s fixation index, \(F\), was used to
determine if levels of heterozygosity within populations are within Hardy-Weinberg expectations (Wright 1951). \( F_{ST} \) values, as well as a number of \( F_{ST} \)-analogs (\( G'_{STest} \), \( \theta'_{ST} \), \( D_{STest} \)), were calculated to assess genetic variation within the different population groups. Comparisons of genetic identity to geographic distance between populations and according to the different genetic sources were determined using Mantel tests of spatial autocorrelation (Mantel 1967). This tested for diversity within the population overall as well as by genetic source (i.e. age-class) or by level of protection (i.e. in protected area vs not) based on total genetic diversity (\( H_T \)) for each polymorphic locus (Nei 1977). Total genetic diversity within (\( H_s \)) and among populations (\( D_{ST} \)) permitted the determination of basic genetic structure over the landscape. This information is of particular interest to land management and conservation organizations (USFWS, BLM, TNC).

For the genus level phylogenetic analysis involving \textit{Arctomecon}, and \textit{Argemone} as an outgroup, the first step was to compare different barcoding regions for their ability to resolve the species-level relationships, and compare these results to those of previous inferences based on morphology, chemistry, and other types of molecular markers. This involved the identification of single nucleotide polymorphisms (SNPs) in microsatellite non-coding gene regions (cpDNA and \textit{nrITS}). The sequences were verified using Sequencher software (ver. 4.10.1, Gene Codes), then aligned in MAFFT (Katoh \textit{et al.} 2002). Evolutionary model testing for each gene region, and phylogenetic analyses were first run in MAFFT and R using the Phangorn library (Schliep 2011). Further phylogenetic analyses were then run under parsimony in TNT (Goloboff \textit{et al.} 2008) to generate trees based on parsimony and maximum-likelihood. Bayesian methods were also employed within BEAST and associated software (Drummond and Rambaut 2007).
**Landscape data**

Mapping software was used to plot all the populations and genotype frequencies, aiding in the spatial analysis and presentation of the data. For this purpose GenGIS (Parks et al. 2009) and ArcGIS were used (ESRI 2009). Geographic data used for mapping some of the reserves was provided by TNC and SITLA (School and Institutional Trust Lands Administration) in order to obtain population locations or determine where reserve boundaries were projected to begin. Each sampled individual received an identification number and the location coordinates were recorded using a handheld global positioning system receiver (GPS; Garmin E-trex) with a precision goal of <4m. In some cases, a single waypoint was recorded for multiple individuals, or multiple waypoints were created to form a population perimeter. Additionally, the condition of the study site (i.e. degree of recreational use, encroaching development etc.) and general observed demographics were recorded. The GPS data was used to generate maps using geographic information system software (GIS; ArcGIS 9), as well as helping to build a predictive model that will be used to investigate possible new localities based on current location and climate data. Landscape level data such as this has proved to be important in addressing questions being pursued by other researchers (e.g. What is the association with cryptobiotic soil crusts? What are the important microhabitat features? What are the continuing threats?). Within these two GIS programs visualization of spatial genetic patterns and statistical analyses of genetic isolation by distance measures, were used to hypothesize further about genetic boundaries, and the processes behind them (Manel et al. 2003).

GIS data layers were available online through the Nevada Natural Heritage Program (http://heritage.nv.gov) and the Utah GIS Portal (http://gis.utah.gov), and include soil, elevation, drainage, and vegetation layers. Climate variables were obtained from WorldClim (Hijmans et al. 2005) and implemented in MaxEnt (ver. 3.1; Phillips et al. 2004, 2006) in order to determine
a basic species distribution model and map. Searches for documented historical populations, within the urban settings of St. George, UT, and Las Vegas, NV, provided a rough estimate of the amount of habitat that has been lost over time, which was of interest to the United States Fish and Wildlife Service (USFWS) (personal communication, Barnes 2009). Additionally, searches within potential habitat near known locations were undertaken in order to locate any undocumented populations.

Research Overview

The initial fieldwork to obtain DNA needed to construct the genomic library, generate initial microsatellite markers, and record population locations and conditions was conducted in April 2009. Ecological transects were run at most *Arctomecon humilis* locations (Chapter 2). DNA extractions were performed at NYBG with phylogenetic sequencing and analyses taking place (Chapter 3). Microsatellite development protocols were initiated, with various methods being tested, followed by preliminary genotyping to establish the best set of polymorphic loci (Chapter 4). Permits were obtained and additional collection trips to sample from outlying locations and to increase sampling in Utah, Nevada, Arizona and California were made during 2010-2012 field seasons. Genotyping of all working variable loci among the samples that were consistently amplifying took place in the fall of 2010 to the fall of 2012 (Chapter 5). The USFWS provided funding for lab work and a portion of the field excursions.
Figure 1-1. Known distribution of the three *Arctomecon* species
Figure 1-2. Flowers (A-C), Capsules (D-F), Inflorescence/Rosettes (G-I) of *A. merriamii*, *A. californica*, and *A. humilis*. 
Figure 1-3. Plant morphotypes of *Arctomecon humilis*: A) small leaf, small rosettes B) larger leaf and larger rosettes. *A. californica* C) large leaf and large rosette D) small leaves and rosettes. (As described by Susan Meyer, personal communication 2009).
Figure 1-4. A. Narrowly ovate *Arctomecon humilis* seed with elaiosome shown running along its length. B. Whole seed showing the elaiosome orientation. C. Magnified elaiosome.
Figure 1-5. Examples of habitat loss due to off-road vehicles, a comparison from the mid-1980’s (A and C) to 2004 (B and D). Photo credit David Wallace, compiled by Tony Frates for the Utah Native Plant Society, 2004.
Table 1-1 Life history traits for each species (Griswold et al. 2011, Nelson and Welsh 1993, TNC 2007a).

<table>
<thead>
<tr>
<th></th>
<th>A. humilis</th>
<th>A. californica</th>
<th>A. merriamii</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pollinator</strong></td>
<td>Ground nesting bees (Synhalonia sp. (Anthophoridae), Tetralonia sp. (Apidae) and Perdita sp. (Andrenidae)), and honey bee (Apis mellifera (Apidae))</td>
<td>Ground nesting bees, Perdita meconis (Andrenidae), Megandrena enceliae and Coleoptera (Schizopus laetus (Schizopodidae) and Trichochroides sp. (Melyridae))</td>
<td>Perdita mohavensis, P. fallugia, Lassioglossum sisymbrii and an undetermined Lassioglossum (Halictidae)</td>
</tr>
<tr>
<td><strong>Seed dispersal</strong></td>
<td>Ant</td>
<td>Ant</td>
<td>Ant</td>
</tr>
<tr>
<td><strong>Flowering season</strong></td>
<td>Multiple white flowers on each inflorescence bloom April-May</td>
<td>Multiple yellow flowers on each inflorescence bloom April-May</td>
<td>Solitary white flowers bloom April-May</td>
</tr>
<tr>
<td><strong>Ecology</strong></td>
<td>Gypsum soils (27-51%), seeds germinate emasse</td>
<td>Gypsum, gypsic limestone (36-69%), one on saline alluvial remnant, also on sites with high Boron or lithium content.</td>
<td>Not as gypsophilic (2-8%), gypsic limestone, widest distribution in altitude and range, yet populations are sparse</td>
</tr>
<tr>
<td><strong>Chemistry</strong></td>
<td>14 unique alkaloids</td>
<td>8 unique alkaloids</td>
<td>5 unique alkaloids</td>
</tr>
<tr>
<td><strong>Longevity</strong></td>
<td>5-6 years</td>
<td>4-5 years</td>
<td>4-5 years</td>
</tr>
<tr>
<td><strong>Fecundity</strong></td>
<td>Avg. 34 ovules/ovary, high pollination and seed fill rates (&gt;80%, &gt;50%, respectively)</td>
<td>Capsules contain 100-160 seeds, average of 13,900 per plant</td>
<td>Many small seeds per capsule</td>
</tr>
<tr>
<td><strong>Mortality</strong></td>
<td>High seedling mortality, pop. maintained by long-lived seeds persistent in seed bank</td>
<td>Estimated 1 in 200 seedlings survive to flower. Undergoes pop. Dormancy, persistent seed bank</td>
<td>39% seedling mortality</td>
</tr>
<tr>
<td><strong>Recruitment</strong></td>
<td>Drastic year-to-year fluctuations, with seeds coming out of dormancy over an extended period of time.</td>
<td>Drastic year-to-year fluctuations, with seeds coming out of dormancy over an extended period of time.</td>
<td>Seedlings germinate emasse</td>
</tr>
<tr>
<td><strong>Reproduction</strong></td>
<td>Mixed mating system, more successful when out-crossing</td>
<td>Self-incompatible, requires pollinator</td>
<td>Out-crosses and self-pollinates</td>
</tr>
</tbody>
</table>
Table 1-2 Species distribution, population status, and level of protection (TNC 2007a, Moorefield 2001a,b).

<table>
<thead>
<tr>
<th>Species</th>
<th>Sites</th>
<th>Pop. groups</th>
<th>Protected sites</th>
<th>Extirpated populations</th>
<th># of Plants</th>
<th>Area</th>
<th>General Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. humilis</td>
<td>11</td>
<td>9-13</td>
<td>~80%</td>
<td>~20%</td>
<td>~15,000, though some populations have fewer than 100 individuals</td>
<td>~4,500 acres</td>
<td>Isolated to within a 15 km radius of St. George, Utah</td>
</tr>
<tr>
<td>A. californica</td>
<td>174</td>
<td>13</td>
<td>~44%</td>
<td>Around Las Vegas, others inundated by Lake Mead</td>
<td>Populations with 1000-70,000 individuals; total ~830,000 plants.</td>
<td>&gt;39,500 acres</td>
<td>From Las Vegas Valley east to Northern perimeter of Lake Mead, including sites in Arizona near Lake Mead and the Grand Canyon</td>
</tr>
<tr>
<td>A. merriamii</td>
<td>135</td>
<td>33</td>
<td>~60%</td>
<td>Losses around Las Vegas, others unknown</td>
<td>~20,000 in small populations with limited extent (65% on Nellis Air Force Base in 1993)</td>
<td>&gt;9,740 acres</td>
<td>Las Vegas Valley, northwest Clark County and adjacent Nye and Lincoln Counties. In California, San Bernardino and Inyo Counties.</td>
</tr>
</tbody>
</table>
Chapter 2: *Arctomecon* Distribution and Community Structure at *A. humilis* Collection Sites.

Introduction

The distribution of the genus *Arctomecon* (Papaveraceae) is largely confined to the northern portion of the Mojave Desert, and all three species inhabit gypsiferous soils (Figure 2-1). Gypsum soils in general contain higher amounts of sulfur, calcium and soluble salts, while they are lower in magnesium (Sheldon 1994), and are sparsely vegetated especially in arid environments like the Mojave. Of the four major deserts in the United States, the Mojave Desert is the oldest, and it contains some of the driest habitats in North America. It is of considerable interest in terms of global climate change because it reflects the dynamics occurring throughout the most arid regions of the world (Smith *et al.* 1997). The geological development of this region is closely tied to the overall flux of the continents and the physical origin of the landscape. Numerous uniquely evolved organisms, found nowhere else on Earth, are supported by the unusual conditions of this habitat. The famed Death Valley, one of the driest, lowest, and hottest points on this earth, is located in the Mojave Desert, and the distribution of *A. merriamii* is located within this extreme environment. Interannual rainfall is highly variable in the Mojave Desert (Hereford *et al.* 2006), which can lead to large fluctuations in species composition and affect the establishment of new seedling cohorts.

The dominant landscape found throughout 70% of the Mojave consists of *Larrea tridentata* (Creosote bush) and *Ambrosia dumosa* (white bursage) vegetation (Schwinning and Hooten 2009). It has been estimated that 33% of the standing biomass of the Mojave Desert is composed of just these two species (Turner and Randall 1989). The frequency of other members within this community alliance will fluctuate, but typically includes *Ephedra* spp., *Atriplex*
confertifolia, Krameria erecta and Lycium pallidum. The distribution pattern of the shrub species illustrates the relatively low diversity over the vast majority of the Mojave. Furthermore, the distribution of perennial forbs throughout the landscape is relatively sparse. This is especially the case at sites supporting Arctomecon, which are generally very sparsely vegetated, harsh habitats that few organisms have been able to carve out an evolutionarily successful niche. Thus the Mojave Desert is home to a number of endangered species, particularly where human development is affecting rare communities and habitat types.

The White bear-poppy, Arctomecon merriamii, is distributed throughout the northern Mojave Desert Ecoregion from Death Valley, California to the Las Vegas Valley, Nevada. According to a recent Nature Conservancy publication, there are 33 patchy localities of the species that can be grouped into seven general geographic areas, with the largest populations found within the Nellis Air Force Base and Desert National Wildlife Refuge (TNC 2007a). The remaining population sites are generally small, limited in extent, and thought to be in slow decline especially in the Las Vegas Valley (Moorefield 2001b). The sites supporting A. merriamii all contain basic soils; alkaline clay or sand, gypsum, calcareous gravels, and carbonate rock (Morefield 2001b). They are found at elevations ranging from 538-2620m (TNC 2007a). The typical community associates include the shrubs; Atriplex confertifolia (shadscale; Chenopodiaceae), Coleogyne ramosissima (blackbrush; Rosaceae), Ephedra torreyana (Mormon tea; Ephedraceae), Lepidium fremontii (pepperweed; Brassicaceae) and Larrea tridentata (creosote; Zygophyllaceae).

The Las Vegas bear-poppy, Arctomecon californica, has a distribution ranging throughout Clark County, Nevada, from within the Las Vegas city limits then east just beyond Lake Mead National Recreation Area. Several populations are also found in Arizona on the
south side of Lake Mead and crossing into the Colorado Plateau ecoregion in the lower Grand Canyon. According to TNC rare plant assessment (TNC 2007a), there are thirteen basic populations that can be grouped into five geographic clusters, with the majority of plants located along the east side of Las Vegas throughout the northern shore of Lake Mead. The substrates supporting *A. californica* include soils with gypsum content between 36-69% (Mistretta *et al.* 1996), but are also known to occur on soils with high levels of boron and lithium (Meyer 1987). The soils are sparsely vegetated with gypsum tolerant species including *Atriplex confertifolia*, *Ephedra torreyana*, *Lepidium fremontii*, and *Petalonyx parryi*. Many sites have high levels of cryptogamic soil crusts as well as abiotic gypsum crust (TNC 2007a). Notably, one survey of *A. californica* found that sites in Arizona tended to be openly vegetated and contained gravelly soils of limestone origin, unlike the Nevada sites with gypsiferous clay. In Nevada, *A. californica* typically inhabits six different soil formation horizons; Las Vegas, Muddy Creek, Thumb, Chinle, Moenkopi and Kaibab (Meyer 1980). According to long-term demography plots, *A. californica* is known to follow the ‘pulse-reserve’ strategy typical of arid desert plants, in which population numbers will fluctuate drastically with short-lived perennials and rare seedling establishment events (Reynolds *et al.* 2004, personal communication Meyer).

The Dwarf bear-poppy, *Arctomecon humilis*, has the narrowest distribution within the genus and is found on the fewest soil horizons. This species occurs within a 15 km radius of St. George, UT, in Washington County. In nearly all cases the species is confined to low gypsum hills and substrates derived from the Moenkopi formation, particularly the Upper and Middle Red member and Shnabkaib member (Nelson and Harper 1991). The study by Nelson and Harper (1991) characterized habitat requirements for the species, measured by soil properties, composition of nine biogenic elements, pH, and micronutrients. They found that within-site
variation for most variables was as great as between sites, and that no site was chemically
distinct from the others (Nelson and Harper 1991). Gypsum content ranged from 27-51%.
Their findings supported previous work done on gypsum soils in that endemism on gypsum is not
likely due to chemical composition, but rather available moisture (Meyer 1986, Nelson and
Harper 1991). In fact, evidence suggests that species that have evolved a tolerance to gypsum
toxicity and soil crust problems may benefit from higher water content in early-summer
gypsiferous soils, in comparison to other desert soils types (Meyer 1986).

Since *Arctomecon humilis* is so closely associated with an edaphic archipelago, distinct
population sites or localities have been easy to identify. In the Dwarf bear-poppy recovery plan
(USFWS 1985) there were seven large and five small sub-populations identified. Typical
community associates include *Lepidium fremontii* (Fremont’s pepperplant), *Atriplex confertifolia*
(shadscale), *Ephedra torryana* (Torrey’s ephedra), *Hymenoclea salsola* (burrowbrush) and
Nelson and Harper (1991) collected their community transect data and absolute living cover data
at two populations, Red Bluff and Price Hills. This provided a comparison to the current
community structure based on point centered quarter (PCQ) transects and nested-frequency data.

In order to conduct genetic research on this genus, all sites of *Arctomecon humilis* were
sampled, and representative sites across the entire geographic range of *A. californica* and *A.
merriamii* were sampled. The tissue sampling categories that were used consisted of the above
ground genetic source (the current plant population) as reproductively mature (producing flowers
or fruit), vegetative (non-flowering), and seedlings (first-year growth). Variation had been shown
between these broad classes within populations, as defined by Van Buren and Harper (1996) and
Allphin et al. (1998). In addition, to understand the variation in plant community structure at sites of *A. humilis*, PCQ transects were set up at all of the larger populations.

**Methods**

*Acquiring permits*

Since all three *Arctomecon* species are either endangered, vulnerable or are located on protected areas, tissue collection permits had to be obtained. This included a permit to collect the endangered *A. humilis* at all known locations in the state of Utah, through the USFWS (Appendix A). As a condition of working with *A. humilis* it was agreed that no destructive sampling of the individual plants would take place (i.e. uprooting plants or excessive leaf damage to seedlings). Permits were also obtained for the *A. californica* population in the Grand Canyon National Park, and for *A. merriamii* in Death Valley National Park, Ash Meadows National Wildlife Refuge, and the Desert National Wildlife Refuge. Additionally, by acquiring a general collection permit through the Nevada Natural Heritage Program, access to some *Arctomecon* sites was made more readily available throughout Nevada, primarily on TNC reserve and the private property of the Las Vegas Airport. Permits were not obtained for Lake Mead National Recreational Area because, as they stated, they were already running their own long-term demographic and ecological transects and did not want further disturbance of the soils and crusts at those sites (Newton, personal communication 2010). The overall sampling goal was to collect leaf tissue from at least 30 individuals at all of the *A. humilis* sites, as well as multiple sites of the other two species from across their more extensive geographic range.

*Sampling*

In order to collect leaf tissue samples for DNA extraction, an initial collection trip was made in April 2009 in which limited sampling took place. Each population site of *Arctomecon*
*humilis* and numerous population sites of *A. californica* and *A. merriamii* were visited and surveyed for GIS mapping. By collecting GPS waypoints at each location, and running the presence data along with climate variables from the WorldClim database (Hijmans *et al.* 2005), a basic species distribution map and predictive map was generated by MaxEnt (Phillips *et al.* 2004, Phillips *et al.* 2006). This was done to assist in the location of unknown populations.

A return trip to each site was made during the 2010 field season in which comprehensive sampling was conducted. During the 2011 and 2012 field seasons outlying populations of genetic interest were visited and sampled, as well as supplementary sampling in previously visited sites. There is one *Arctomecon californica* population group, composed of three to four sites in the Grand Canyon area that has been noted as being morphologically and ecologically distinct (personal communication Harper, Van Buren and Meyer 2009). One of these populations was visited and sampled during these follow-up trips. The Death Valley populations were also visited in 2012 in order to sample the *A. merriamii* populations that are distributed furthest west. The site visits involved locating known populations as well as exploring likely terrain and soil types. Once a site was identified and located, perimeter tracks were created using a handheld GPS unit. Tracks and waypoints were downloaded daily using DNR Garmin GPS Application (Minnesota DNR, version 5.4.1) and uploaded into ArcGIS (version 9.3).

Care was taken to ensure that leaf samples were free from any fungus, other infection, or dirt. Clippers were surface sterilized with a mild bleach solution between each sampled plant. Leaf material was placed in numbered polyethylene bags containing silica gel beads as a desiccant. Leaf tissue was transported to NYBG for DNA extraction. Since the USFWS permit limited destructive sampling, all individuals that were used for tissue sampling had digital images taken in order to provide photographic vouchers for future reference.
The aim was to collect leaf tissue (1-2 leaves) from a minimum of 30 individuals in each population with equal representation from multiple sources representing the entire genetic variation of each population. Temporary transects were set up in order to ensure that the sampling of individuals was randomized. Additionally, PCQ transects were used to collect ecological and plant community data at nine of the *Arctomecon humilis* sites.

**Transects**

Plants were randomly selected for leaf tissue collection along a 100m transect. The transects were also used as PCQ plots (Cottam and Curtis 1956), largely following Mitchell (2001) to facilitate the collection of ecological data (Figure 2-2). Community composition, relative density, relative cover, relative frequency, importance values and absolute density were calculated from the point-to-plant center data. Importance values represent a measure of the relative dominance of a species by summing the relative frequency, relative density, and the relative basal area or cover of a species in a particular community. The measure of absolute density is important from an ecological and conservation standpoint, and could be combined with other demographic data to aid in landscape management activities. A nested-frequency quadrat frame (Smith *et al.* 1987) was also centered on each point along each transect in order to determine coverage and relative frequency.

In order to avoid counting the same plant in quarters of adjacent transects, there was a minimum of 10 meters between each point. Additional transects were created and sampled as the population deemed necessary. The collector walked along each transect and at predetermined random points stopped, and located the plant nearest the point within each quarter (quarters are created by the main transect and an imaginary line perpendicular to the transect). Distances from the plant in each quarter to the transect point were measured with a second
measuring tape. This method should adequately reflect the genetic variation across the population and should avoid false indications of isolation by distance. The minimum distance between two points along transects was 10 m. This is well outside the experimentally observed ant-dispersed-seed transport distances of 60 cm (Hickerson and Wolf 1998).

Transects served to catalog the presence of the vascular plant community at nine collection sites throughout known *Arctomecon humilis* habitat. This included populations at White Dome, Price Hills, Boomer Hill, Warner Ridge, Beehive Dome, Shinob Kibe, Red Bluff, and two fragments of what used to be Atkinville, which were called Sun River and Red Wash in this study. Points along the transect were selected using a random number generator, but the transects themselves were situated in areas with a relatively high density of *A. humilis* plants in order to ensure the cataloguing occurred within proper habitat.

**Results**

**Sampling Locations**

Leaf samples from all three *Arctomecon* species were made with an attempt to sample from at least 30 individuals at each site, including leaf tissue from a variety of size classes and morphologies along the transects. Variation in leaf morphology can also exist in winter foliage versus spring foliage of the rosettes, so caution was taken when determining age class in different seasons (personal communication, Van Buren 2009).

A total of 1176 plants were sampled, photographed and catalogued. This consisted of 406 *Arctomecon humilis*, 540 *A. californica*, 225 *A. merriamii* and 5 individuals of other poppy species to serve as outgroups (*Argemone corymbosa*, *Meconopsis cambrica* and *Eschscholzia glyptosperma*). DNA extractions were performed for 949 of these samples; 367 *A. humilis*, 364 *A. californica*, 213 *A. merriamii* and the 5 outgroup individuals. Some DNA extractions were
not performed because it was determined that certain population sites may be over-represented in comparison to others. Fewer than 30 individuals were located at 10 sites, while the 25 other sites had at least 30 samples collected. Samples obtained from a single location ranged from a minimum of 4 individuals, to a maximum of 55 individuals. Low counts were due to an inability to locate additional plants in an isolated site, or finding a population with many seedlings but few larger plants. High counts were made at locations with large populations spread over a vast area, with more samples collected as the extent of the site was realized, this was done to ensure sampling from across the entire genetic range of the site.

For organizational and labeling purposes, the collection locations or ‘sites’ were synonymous with populations, until further analysis was conducted. Site names were determined based on previous studies and literature, or given a location name if one did not already exist. Additionally, some sites are very close geographically, yet were still given separate site names if the individuals were located on a distinct substrate or soil outcrop in comparison to the next nearest collection site. The original number of sampling locations was thirteen *Arctomecon humilis* sites, seventeen *A. californica* sites, and seven *A. merriamii* sites (Table 2-1; Figures 2-3,4,5). It should be noted that the Grand Canyon population sites are technically not in the Mojave Desert ecoregion, but rather in the adjacent Colorado Plateau ecoregion (TNC 2007a). The community and habitat, as well as the basic morphology of the plants, exhibit noticeable differences as compared to other *A. californica* population sites.

*Distribution map*

The distribution map produced by MaxEnt (Phillips *et al.* 2006) using verified location data in combination with bioclimatic variables identified the distributions of all three species, however, predicted areas of highly likely distribution were only identified for *Arctomecon*
*merriamii* (Figure 2-6). Searches were carried out in the areas identified, but no additional populations were located. When visiting the predicted sites I could not visually identify promising habitat, nor could I devote significant time to this endeavor. This analysis would have been more powerful with the input of soil data, but a suitable fine-scale dataset could not be obtained. The location waypoints of all *A. humilis* populations, as well as locations that were checked but no plants were located (negative values) were also provided to a United States Geological Survey (USGS) researcher in order to aid in the construction of a more detailed predictive habitat map in hopes of locating new sub-populations (Bowker, USGS 2012).

**Community Composition at A. humilis sites**

Transects with PCQ and nested-frequency were carried out at nine of the sites originally identified as population groups according to the USFWS (1985), although some of their locations have since become fragmented or lost due to urban development and habitat degradation. The sites furthest from the city center show less damage from off-road vehicles, and the sites are largely fenced by the BLM. These include Boomer Hill on the west, Beehive Dome in the southeast and Warner Ridge on the eastside. The sites with residential development nearby are Red Bluff, Price Hills and Shinob Kibe, all of which are currently fenced as habitat reserves. However, there is active construction directly along the fence line of the Shinob Kibe reserves now owned by The Nature Conservancy. The other area recently purchased by TNC is White Dome, south of St. George, but prior to this it had considerable damage by off-road vehicles. Lastly, the fragments of what used to be the Atkinville population (USFWS 1985), have primarily been impacted by the interstate highway I-15 being constructed directly through poppy habitat.
There were a total of thirty-one biotic associates co-occurring near *Arctomecon humilis* at the nine locations as measured at 136 transect points. The number of species at a single site ranged from a low of nine species at Red Bluff, to a total of seventeen species found at Red Wash, Price Hills, and Beehive Dome (Table 2-2). Although Red Bluff had the smallest number of community associates it was also the only site without introduced species found within the sample plots and significantly more cryptobiotic cover (73.5%, Table 2-3) than at any other site. The Boomer Hill site proved to be the most dissimilar in comparison to the other sites, being the only location with 0% cryptobiotic soil, lacking *Atriplex confertifolia*, and having the sparsest distribution of *Arctomecon humilis* plants. Based on the PCQ quadrat data, importance values were calculated across all sites (Table 2-4 to Table 2-7). *Atriplex confertifolia* had the highest importance value (70.97, Table 2-4), followed closely by *Ephedra torreyana* (70.27). The next highest values, apart from *Arctomecon humilis* (30.02), were two species of *Eriogonum*, *E. cernuum* and *E. inflatum* (21.93 and 21.48, respectively).

According to a Jaccard similarity coefficient analysis, based on the presence of species in the nested frequency plots at all *Arctomecon humilis* sites, it was found that *Atriplex* and *Ephedra* were associated with the presence of *Arctomecon* (Figures 2-7 and 2-8, p < 0.0001). Generally, it would be expected that localities where these plants are present there may be a higher likelihood of locating *Arctomecon humilis*. However, due to the high specificity of *Arctomecon* to particular substrates, it is difficult to characterize a set of co-occurring species that are not also located at a number of other locations that *Arctomecon* is not found. Nelson and Harper (1991) analyzed the soils at another location near St. George, Pugatory Point, which seemed to have all the characteristics that typically support *Arctomecon*. They did not detect significant differences between sites, other than a small number of *Larrea tridentata*, and even
proposed that it be maintained as potential habitat for the poppies (Nelson and Harper 1991). Unfortunately, this site has since been degraded by development and construction. Regardless, the presence/absence data of other species is not always a good predictor of rare species. In fact, the Boomer Hill site did not have either of these species detected in the PCQ plots or the nested frequency plots. One shrub species, *Ambrosia salsola*, was found in many of the nested frequency plots, but was not detected in the PCQ plots because it was never the closest plant to the point, a small number of forbes undetected by PCQ were also found in the nested frequency plots.

The average cover of cryptobiotic soils across all the study sites was 38.6%, with a range from 0-73.5%. It should be noted that Harper and Nelson (1991) ran similar transects at two sites (Red Bluff and Price Hills, both included in the current study) and found that non-vascular biota (lichens and mosses) made up 84% or more of total living cover at each site. According to my observations, Price Hills had abundant cryptobiotic cover in the center of the reserve, as did Webb Hill, just north of Price and at one time contiguous with it. In the current study, the cryptobiotic cover at Price Hills was 37.1%.

Harper and Nelson (1991) found that shrub species at *Arctomecon humilis* sites were similar to each other except *Lepidium fremontii* was significantly more abundant at Price Hills. They noted the two typical invasive grass species, *Bromus tectorum* and *B. rubens*, were found at most sites but were in low quantities, generally only inhabiting the base of shrubs or disturbed areas (i.e. old foot traffic, margins of trails or tire tracks). The results of the current study also show a low abundance of introduced species at most sites, except at Shinob Kibe. This locality is separated from the others, with significant levels of construction surrounding the site.
Figure 2-1. Maps encompassing the entire global distribution of *Arctomecon* within the Mojave Desert and Colorado Plateau. A. The range covered by all species as located during the current study. B. Collection sites for *Arctomecon merriamii* that were included in the study. C. Collection sites for *Arctomecon californica* that were included in the study. D. Collection sites for *Arctomecon humilis* that were included in the study.
Figure 2- 2. Location information from a 2003 survey of endangered plants conducted for the School and Institutional Trust Lands Administration. As plants were located their position was recorded using a handheld GPS device and this information was used to create comprehensive distribution maps (similar to Map A). This map, of the White Dome population of *Arctomecon humilis* is certainly more thorough than known distributions of most other populations. However, it does give insight into the clustered distribution of the populations in general.

Map A. A subset of the White Dome population, southeast of St. George, Utah, depicting the number of plants as variously sized circles.
Figure 2-2 Transect Continued. Map B depicting all locations based on plant density. Map C is an example of a possible transect through this population, it is currently depicting only one point, but in practice each had multiple points.

Map B. This is a Point density plot of all the *Arctomecon humilis* locations in the White Dome survey area.

Map C. Hypothetical transects through a small section of White Dome. The bottom transect has a perpendicular dotted line to show the creation of the four quarters.
Figure 2-3. Sampling locations of *Arctomecon humilis*
Figure 2-4. Sampling locations of *Arctomecon merriamii*
Figure 2-5. Sampling locations of *Arctomecon californica*
Figure 2-6. MaxEnt predictive modeling of species distributions based on worldclim climate variables for *Arctomecon merriamii*, *A. californica* and *A. humilis*. 
Figure 2- 7. Observed Jaccard similarity coefficient (J= 0.196, p-value<0.0001) against the randomized null distribution for *Ephedra torreyana* co-occurring with *Arctomecon.*
Figure 2-8. Observed Jaccard similarity coefficient ($J=0.208$, $p$-value$<0.0001$) against the randomized null distribution for *Atriplex confertifolia* co-occurring with *Arctomecon* at all locations.
Table 2-1. Arctomecon collection sites, currently being treated as individual populations for locus analyses.

<table>
<thead>
<tr>
<th>Species</th>
<th>Collection locations</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. californica</em></td>
<td>Apex Road, Patch, Red Patch, Lime Ridge, LV Airport, TNC, Rainbow Canyon, LV Dunes, Burro, South Burro, Whitney, Lost, Eagle Mine, Stash, Borax, Pabco, Bitter</td>
</tr>
<tr>
<td><em>A. merriamii</em></td>
<td>Ash Meadows, Horse, Kane Springs, Calico, Race Track, Titus Canyon, Scotty’s Castle</td>
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Table 2. The total species richness at all *Arctomecon humilis* sites based on PCQ transects. There were 136 random points at nine collection locations (continued onto the following page). Number of species per site ranged from nine to seventeen.

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<th>Price Hills IV</th>
<th>Red Wash IV</th>
<th>Boomer Hill IV</th>
<th>Warner Ridge IV</th>
<th>Beehive Dome IV</th>
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| Cryptobiotic             |       |               |        | 38.7    | 40.0       | 7.5        | 37.1        | 11.4      | 0.0      | 54.5        | 17.6         | 58.6        | 73.5       |
| Litter                   |       |               |        | 1.4     | 1.2        | 0.0        | 1.1         | 7.5       | 0.0      | 0.9         | 1.4          | 1.0         | 0.7        |
| Rock >2cm                |       |               |        | 13.4    | 20.5       | 10.9       | 9.7         | 36.8      | 6.1      | 10.1        | 12.9         | 4.2         | 6.4        |
| Pavement 2mm-2cm         |       |               |        | 4.4     | 3.3        | 6.3        | 4.9         | 3.8       | 9.6      | 2.7         | 9.2          | 0.0         | 1.6        |
| Soil                     |       |               |        | 42.1    | 34.9       | 75.3       | 47.4        | 40.5      | 84.3     | 31.9        | 59.0         | 36.2        | 17.9       |
Table 2-4 The twelve species with highest ranked importance values based on PCQ analysis (136 points).

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<th>Species</th>
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Chapter 3. Phylogeny of *Arctomecon*

**Introduction.**

*Arctomecon* Torr. & Frem. is one of the desert-poppy genera within the sub-family Papaveroideae found in the western United States. Other members of this group include *Argemone* L., *Canbya* Parry ex. A. Gray, *Romneya* Harv. and *Platystemon* Benth. These desert genera are known to have close affinity to one another, and form a monophyletic group apart from the largely Old-World genera of the sub-family (*Papaver* L., *Meconopsis* Vig., *Roemeria* Medik.) (Kadereit *et al.* 1994, Jork and Kadereit 1995, Kadereit *et al.* 1995). While the relationships within the Old-World clade were supported by multiple clear morphological synapomorphies, the New World clade (*Arctomecon, Argemone, Romneya, Canbya, Platystemon, Hesperomecon* and *Meconella*) proved to be problematic due to extremely diverse and unique morphology found within each genus. Studies based on morphology and molecular data, found that the Old World clade was clearly monophyletic (17 synapomorphies), but among the North American desert group there were far more autapomorphies as compared to synapomorphies (Kadereit *et al.* 1994, Hoot *et al.* 1997). A subsequent study using Restriction Fragment Length Polymorphism (RFLP) was able to verify monophyly for the species-rich genus *Argemone*, as well as establish *Arctomecon* as the sister group to *Argemone* (Schwarzbach and Kadereit 1999), but was not able to determine the phylogentic relationships among species within each genus, including those of *Arctomecon*. The authors of these studies explain the lack of resolution being due to rapid diversification and ecological adaptation in arid environments (Schwarzbach and Kadereit 1995, Schwarzbach and Kadereit 1999) and not a consequence of the genetic markers they were using.
*Arctomecon* is an example of a small genus that is morphologically distinct from other closely related genera. The unifying characters found in all three species of the genus include their basic growth form; perennial, with long taproots, woody caudices, and one to many dense rosettes (Nelson and Welsh 1993). They have uniquely cuneate leaves, densely covered by long hairs, with each leaf lobe ending in a claw-like bristle. They possess a non-reticulated seed coat surface and an oil-rich aril, while *Argemone* and *Romneya* both have fine reticulation and lack an aril. The three *Arctomecon* spp. also have unique alkaloid chemistry and are the only known gypsum obligates in the Papaveraceae (Nelson and Welsh 1993, Schwarzbach and Kadereit 1999).

*Arctomecon* is unique when compared to the two most closely related genera, however their morphological autapomorphies are easily evident from one another. For example, the three species differ in flower arrangement on an inflorescence, flower color, whether petals remain or are lost after anthesis, number of locules per ovary, and overall stature of the plant (Nelson and Welsh 1993). While *A. californica* has yellow, 3-merous flowers, caducous petals, and a persistent capsule, *A. humilis* has white, 2-merous flowers, persistent petals, and a capsule that detaches from the plant (Schwarzbach and Kadereit 1995). Both *A. californica* and *A. humilis* have branching inflorescences, while the white, 3-merous flowers of *A. merriamii* are borne on single-flowered peduncles. Pronounced differences in the development of floral structures, branching patterns, and perianth identity such as these have been described as the result of gene duplications for some members of the Papaveraceae (Pabon-Mora, Ambrose and Litt 2012). The taxonomic treatment of the genus noted that the strong morphological differences combined with their geographic isolation suggested that the phylogenetic separation of these three species was not a recent event (Nelson and Welsh 1993).
The use of molecular systematics in studies of closely related species is of particular importance for filling in the “tips” on the angiosperm tree of life (Palmer et al. 2004). Previous molecular studies involving *Arctomecon* have inferred relationships among the genera or species, but only two have included all three *Arctomecon* species. Schwarzbach and Kadereit (1995) included the three *Arctomecon* species in their RFLP study, however they formed a polytomy with *A. californica* and *A. humilis* possessing identical restriction site patterns, and *A. merriamii* exhibiting four autapomorphies. In a later study, designed to resolve the species relationships within *Argemone*, only *Arctomecon humilis* was included as an outgroup and sole representative of the genus so no further information was gleaned for the relationships within *Arctomecon* (Schwarzbach and Kaderiet 1999). Van Buren and Harper (1996) used randomly amplified polymorphic DNA (RAPD) markers and found that *A. californica* and *A. humilis* showed more similarities with each other (23%), than either did with *A. merriamii* (15% and 18%, respectively). While this study provided a good probability of the interspecific relationships, RAPD data must be interpreted cautiously when estimating phylogeny (Van Buren and Harper 1996). RAPD data has been shown to lack consistent reproducibility (Jones et al. 1997, Bagley et al. 2001, Rajput et al. 2006), and should be combined with additional supporting data (Jones et al. 1997, Agarwal et al. 2008).

While the monophyly of *Arctomecon* has never been questioned, other studies have inferred that *A. californica* and *A. merriamii* were more similar and likely derived while *A. humilis* was sister to them according to alkaloid chemistry (Raynie et al. 1991, Nelson and Welsh 1993) and the type of trichomes present (Mistretta et al. 1996). Schwarzbach and Kadereit (1999) stated that *A. humilis* was most derived based on morphology and that *A. californica* is nested within the other two species, citing unpublished data. Two other studies
involving molecular data did not include samples from all three species (Hickerson and Wolf 1998, Allphin et al. 1998). The lack of inclusion of all three species in other studies has likely been due to the small size of the genus and rarity of the species. Additional supporting information about which species is sister to the other two or most divergent that will confirm the RAPD study is still called for (Van Buren and Harper 1996). Additionally, a sub-population group of *A. californica* from the Grand Canyon has been suggested to represent a morphologically, ecologically and geographically distinct population but has never been included in a molecular study (Harper, Phillips and Hodgson, personal communication). The aim in carrying out this portion of the study was to confirm or determine the phylogenetic relationships within *Arctomecon* by sequencing and comparing single nucleotide polymorphism (SNP) data from multiple gene regions that have proven to be phylogenetically informative at the species level or that have been included in DNA barcoding (CBOL Plant Working Group 2009). Obtaining samples from across the range of each species with the inclusion of a representative from the Grand Canyon population was a priority.

Since morphological differences alone were not enough to resolve the New World genera, Schwarzbach and Kadereit (1995) implemented the RFLP study using PCR amplified chloroplast genome fragments. They were able to determine relationships among the genera, but still not among species within each genus. In order to resolve the phylogeny of *Argemone* at the species level they sequenced the internal transcribed spacer (*nrITS*) regions of the nuclear ribosomal DNA (*nrDNA*) with the inclusion of *Arctomecon humilis* as an outgroup (Schwarzbach and Kadereit 1999). Knowing that the *nrITS* region has provided resolution at the intrageneric level in other taxonomic groups (Baldwin 1993, Wojcienchowski et al. 1993) and in the closely related *Argemone*, the *nrITS* region was included in the present study.
The *rbcL* gene was one of the first markers used in a large number of plant phylogenetic studies, demonstrating its utility in comparative sequencing. This region is typically used to determine phylogenies at the tribe or family level and above, or is used in combination with other gene regions, and was not expected to exhibit much variation within the genus. Nevertheless, it was included in this study since it was suggested as an official plant barcode by the CBOL Plant Working Group (2009), and was used among other Papaveraceae species at the NYBG (Pabon-Mora, unpublished). The *matK* gene is a rapidly evolving coding region that was also included here because it has shown promise in phylogenetic studies and as a plant barcode when paired with *rbcL* (Johnson and Soltis 1995, Tate and Simpson 2003, Hilu *et al.* 2008, CBOL Plant Working Group 2009). Additionally, for closely related taxa it has been suggested that the non-coding *trnH-psbA* spacer be used in combination with *rbcL* and *matK* due to its amplification across land plants using a single primer pair and its high variability leading to species discrimination (Shaw *et al.* 2005, Kress and Erickson 2007, Chase *et al.* 2007).

While these regions have proven to be useful for DNA barcoding and species identification, additional non-coding cpDNA regions were included in order to gain more insight into the genetic variability at the population level. For this purpose the *ndhF-rpl32* and *rpl32-trnL* regions that have exhibited high PIC (potentially informative character) values and high rates of variation (Shaw *et al.* 2007) were employed in order to estimate levels of intraspecific diversity. These two regions in combination with *trnH-psbA* were used to differentiate among populations of *Hunnemannia fumarifolia* (Papaveraceae) a poppy endemic to the Chihuahuan Desert and Sierra Madre Oriental (Sosa *et al.* 2009). The inclusion of these regions in the current study were used to test for patterns of isolation by distance, and population differences prior to more in-depth population genetics based on microsatellite markers.
Methods

Taxa and Outgroup Selection

Collection of samples from field locations was conducted primarily during the spring of 2009 and 2010, with collections in outlying populations occurring in 2011 and 2012. A total of 1176 individual plants were sampled for the population level study involving the three Arctomecon species (Chapter 4 and 5). The sampled plants consisted of 406 A. humilis from thirteen locations, 540 A. californica from seventeen locations, and 225 A. merriamii from seven locations. A subset of these samples was used for the phylogenetic study, with fourteen A. humilis, nineteen A. californica, and fourteen A. merriamii specimens being included in the combined phylogenetic tree (Table 3-1). At least one sample from each collection site was included, as these represent the geographic range and habitat diversity of the genus. In addition, three taxa, Argemone corymbosa, Meconopsis cambrica and Eschscholzia glyptosperma served as outgroups. These taxa were selected as outgroups because they are in the Papaveraceae, yet represent increasing levels of divergence from the ingroup. Argemone and Meconopsis are in the same subfamily, Papaveroideae, with Argemone being the sister genus to Arctomecon (Hoot et al. 1997, Schwarzbach and Kadereit 1999). Eschscholzia is a member of a separate subfamily, Eschscholzioideae (Hoot et al. 1997). For the Arctomecon specimens, unique identifiers were associated with the collection locations in order to compare phylogenetic structure with geographic patterns.

Depending on the size of individual plants, one to four fresh leaves were obtained and then immediately placed within a coffee filter and plastic bag with silica gel beads for preservation. Every known locality of Arctomecon humilis was sampled and at least one individual from each site was included in the study. Samples from across the ranges of A.
*californica* and *A. merriamii* were also included from sites representing their geographic distribution, although not every known site was included. For *A. californica*, collection sites included: two within the Las Vegas city limits (TNC reserve and the Airport), five sites just east of the city (Apex, Las Vegas Dunes, Pabco Mine, Rainbow Gardens, Eagle Mine), three sites just outside of the Lake Mead National Recreation Area (Borax Mine, Lost, Bitter Springs), four sites around Gold Butte (Whitney Pocket, Burro range, Lime Ridge, Patch), and a single population in the Grand Canyon National Park (Stash). The Grand Canyon populations have been noted for their divergent morphology, habitat and ecological community by numerous researchers and DNA samples from this locality were made a priority for the phylogenetic study. There are at least two additional populations within the Grand Canyon, found on the Hualapai Reservation, from which I was unable to obtain a permit in time for field collection. Two attempts were made to visit additional populations, but transportation and accessibility problems arose.

Seven different *Arctomecon merriamii* sites were included in the study, with at least two individuals from each site being sequenced. This included three populations from within Death Valley National Park (Scotty’s Castle, Titus Canyon, Race track), which represented the westernmost populations of the species and genus. Collections were also made at Ash Meadows Wildlife Refuge southwest of Death Valley in Nevada, the Desert National Wildlife Refuge (DNWR) and Kane Springs north of Las Vegas, and Calico Basin in the Red Rocks National Park west of Las Vegas. Attempts to locate and collect from four additional *A. merriamii* sites were made, south and west of Las Vegas, but without success. Populations of both *A. californica* and *A. merriamii* have been known to go dormant, only being found within the seed bank, for multiple years until environmental conditions are more favorable (Meyer 1986). Populations within the Nellis Air Force Base were also not accessible to me for sampling due to travel
restrictions on their property grounds, however the large population in the adjacent DNWR was considered a reasonable representative of the area as these largest populations are essentially contiguous with each other.

**DNA Extraction**

All leaf tissue samples were transported to NYBG for storage and further processing. Extraction of DNA from dry leaf tissue followed the Qiagen DNeasy Plant Miniprep protocol (Qiagen Inc., Valencia, CA, USA) or a slightly modified version of this method (Elphinstone et al. 2003, Alexander et al. 2007). Dry leaf tissue was weighed (~0.02 g) and added to a sample tube, where it was pulverized using a ceramic bead and sterile garnet granules. The powdered leaf tissue was then covered with 400 µL of warmed Buffer AP1 and 4 µL of RNase A stock solution then mixed by pipetting. The solution was incubated at 65°C in heat block for at least 10 minutes. Next 130 µL of Buffer AP2 was added to the lysate, then mixed, and incubated on ice for 5 minutes. This mixture was then centrifuged for 5 minutes at 13,000 rpm. The top layer of solution was pipetted to a new tube and centrifuged for an additional 5 minutes at 13,000 rpm and the resulting top layer of supernatant being pipetted into a new tube. Next, ~600 µL (about 1.5x the volume) of Buffer AP3/E was added to the tube and mixed. 650 µL of this mix was then transferred to the DNeasy Mini spin column with collection tube (2 mL) (Qiagen Inc.) and centrifuged for 1 minute at 8000 rpm. The resulting flow through was discarded. Reusing the collection tube by placing the spin column back in and adding another (the remaining) 650µl and repeating the last step (spin and discard flow through). Next the spin column was inserted into a 2 mL collection tube, and 500 µL of Buffer AW was added. This was centrifuged for 1 minute at 13000 rpm, flow through was discarded and this step repeated. Lastly, the empty spin column and collection tube were centrifuged for 2 minutes at 20,000 rpm to dry the column membrane,
making sure there was no residual ethanol left over. The DNeasy Mini spin column was then transferred to a 2 mL centrifuge tube and 100 µL buffer AE pipetted directly onto the DNeasy membrane. This incubated for 5 minutes at room temperature, then centrifuge 1 minute at 8000 rpm. If necessary, an additional ethanol precipitation and purification step was conducted.

**PCR Protocols**

The nrDNA ITS region and five cpDNA regions were sequenced including two genes (\textit{rbcL} and \textit{matK}) and three non-coding gene regions (\textit{ndhF-rpl32}, \textit{rpl32-trnL}, \textit{trnH-psbA}) for the three \textit{Arctomecon} species as well as three different outgroups (Table 3-1). PCR amplifications were performed in 25 µL reactions. Each reaction contained 9.3 µL of autoclaved ion-exchanged water, 2.5 µL of dNTP mixture (2.5 mM stock of each dNTP), 2.5 µL of bovine serum albumin (BSA; 0.25 µg µL\textsuperscript{-1} stock), 2.5 µL of red buffer [200 mm Tris pH 8.8, 100 mM KCl, 100 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 20 mm MgSO\textsubscript{4}·7H\textsubscript{2}O, 1 % (v/v) Triton X-100, 50 % (w/v) sucrose, 0.25 % (w/v) cresol red], 5 µL Q-Solution (betaine, 1.2 M stock), 1 µL of each primer (0.67 µM final concentration), 0.2 µL of Taq polymerase (GenScript USA Inc., Piscataway, NJ, USA) and 1 µL of DNA.

The \textit{nrITS} region was amplified and sequenced using primers ITS-P5 (Eckenrode \textit{et al.} 1985) and ITS-B (Blattner and Kadereit 1999) (Table 3-2). The chloroplast gene \textit{matK} was amplified and sequenced using \textit{matK} 390F and \textit{matK} 1326R (Cuenoud \textit{et al.} 2002) while \textit{rbcL} was amplified using two pairs, \textit{rbcL} 1F/724R and 636F/1368R (Lindquist and Albert 2002). The cpDNA spacer regions were \textit{ndhF-rpl32}, \textit{rpl32-trnL} (Shaw \textit{et al.} 2007) and \textit{psbA} (Sang \textit{et al.} 1997)/\textit{trnH} (Tate and Simpson 2003), following parameters in Shaw (2005).

PCR reactions were run on an Eppendorf vapo.protect Mastercycler pro S with each primer set having a different set of cycling parameters. The \textit{nrITS} parameters were: initial
denaturing for 2 min at 94°C, followed by 38 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension of 5 min at 72°C; *matK*— initial denaturing for 2 min at 94°C, followed by 26 cycles of 94°C for 1 min, 48°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 7 min; *rbcL*— initial denaturing for 2 min at 95°C, followed by 34 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 7 min. The cpDNA spacers: *trnH-psbA* — initial denaturing for 2 min at 95°C, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, followed by a final extension of 5 min at 72°C; *ndhF-rpl32*— initial denaturing for 5 min at 80°C, followed by 30 cycles of 95°C for 1 min, 49°C for 1 min, and 65°C for 4 min, followed by a final extension of 5 min at 65°C; *rpl32-trnL*— initial denaturing for 5 min at 80°C, followed by 30 cycles of 95°C for 1 min, 52°C for 1 min, and 65°C for 4 min, followed by a final extension of 5 min at 65°C. Products from the PCR for all gene regions were sent to the University of Washington’s Department of Genome Sciences, High-Throughput Genomics Unit Sequencing Center.

**Contig Assembly and Sequence Editing**

Sequences were uploaded into Sequencher 4.10.1 (GeneCodes Corp., Ann Arbor, Michigan) and assembled into contigs based on unique identifier handles. Contigs were then cleaned up and any necessary peak calls were made. The sequences were exported from Sequencher as fasta files and aligned using the web-based version of MAFFT (G-INS-i) for each gene region (Katoh *et al.* 2002, Katoh *et al.* 2005). Aligned sequence files were then used or uploaded for use in analysis programs.

The DNA sequences will be submitted as DNA barcodes to the NCBI (National Center for Biotechnology Information) GenBank database (Benson *et al.* 2013). A Perl script was used to compare aligned sequences and determine the nucleotide sites that are variable for each region, and each species (Little, unpublished, Appendix D varSequences.pl).
Phylogenetic Analysis

The genus level phylogenetic analysis of Arctomecon, with Argemone, Meconopsis, and Eschscholzia as outgroups was run using both parsimony (TNT) and Bayesian methods (BEAST). Some researchers prefer parsimony approaches to phylogeny inference because they do not make assumptions about models of molecular evolution and find the tree that requires the fewest evolutionary changes. However, it has been noted that parsimony methods can fail when shared homoplasies lead to the attraction of distantly related taxa on a phylogeny due to unaccounted for multiple substitutions at individual sites in the alignment, a problem commonly referred to as long branch attraction (LBA - Felsenstein, 1978). Bayesian methods use models of sequence evolution to account for multiple substitutions and thus do not suffer from LBA. The individual gene trees were generated using a TNT parsimony analysis script, in order to obtain the bootstrap consensus trees. The TNT analyses implemented a parsimony ratchet tree search with TBR branch swapping. One thousand replicate datasets were used for bootstrapping and inferring node support values. The sequences of all six regions were then combined for the greatest number of samples available in each species (refer to Table 3-1). The combined data set was run following the same TNT protocol and parameters as the individual gene trees (1000 replicate datasets). The phylogeny and species tree were inferred under maximum parsimony. Trees were finalized using Mesquite v. 2.75 (Maddison and Maddison 2011).

The Bayesian analyses were run in BEAST (Drummond et al. 2007) and *BEAST (‘StarBeast’; Heled and Drummond 2010). The BEAST platform uses a Bayesian MCMC analysis of the sequences over the tree space and weights each potential tree on its posterior probability. The programs Tracer (Rambaut and Drummond 2007), FigTree and DensiTree (Bouckaert 2010) were used to visualize the tree topologies and Bayesian analysis. The program
Beast infers the species tree topology from multiple gene regions based on samples of multiple individuals from closely related species. The separate fasta alignments were converted to Nexus format and uploaded separately as six partitions into BEAUti (Bayesian Evolutionary Analysis Utility), where the evolutionary model and MCMC analysis parameters are set up. In BEAUti the site model was set up with a substitution model HKY, empirical base frequencies, Gamma + Invariant. The clock models were uncorrelated, MCMC length of 10,000,000 logging every 1,000 trees. The file created by BEAUti was then run in BEAST implementing the stochastic MCMC algorithm. The resulting trace file from the BEAST run was imported to TracerThe tree output file was then imported to Tree Annotator to determine the maximum clade credibility tree, and trees were visualized in FigTree and DensiTree.

Results
Phylogenetic Analyses
All gene regions amplified easily according to the referenced PCR protocols, and all sequences amplified the first time except for a single Meconopsis sample. The ndh-rpl32 region amplified in Meconopsis with a slightly altered protocol, but the sequence still came back unclear and thus this outgroup was not included in all of the analyses. According to the parsimony analysis run in TNT on both the individual trees and the concatenated gene regions there was strong support for a robust species delineation, with Arctomecon californica and A. humilis being sister to each other and more recently derived as compared to A. merriamii (Figures 3-1 to 3-7, Table 3-3). As expected the matK and rbcL regions were not highly variable (Table 3-1 contains the number of informative characters per region). They were both able to determine the monophyly of A. merriamii, but could not delineate between A. humilis and A. californica according to the majority-consensus trees. The nrITS, and ndh-rpl32 were the most
informative and highly variable regions. However, in the majority-consensus tree (Figure 3-1) based on the concatenated sequences, the bootstrap support for a subset of *A. humilis* was 69.8 percent and populations of *A. merriamii* were at 63 percent.

The original analysis of *trnH-psbA* sequences had grouped five populations of *Arctomecon humilis* and four populations of *A. merriamii* with *Argemone* (Figure 3-4). While the *trnH-psbA* region has been suggested as a candidate for DNA barcoding among plant species it is also well known to have frequent inversions (Whitlock *et al.* 2010). This has been noted as a potential problem for this region because it has been identified among many interspecific phylogenetic studies of angiosperms, but has not been well documented intraspecifically (Whitlock *et al.* 2010). Within two of the three *Arctomecon* species intraspecific inversions are present, none were detected in *A. californica*. This analysis demonstrates the potential errors that could arise from using only the combined barcoding regions of *rbcL*, *matK*, and *trnH-psbA*. The inverted region was a 26 basepairs in length in both *A. merriamii* and *A. humilis* with a single base pair difference between the inversion of the two species (Figure 3-8).

After identifying the sequences with the inversion, an alteration of the sequences was performed by removing and replacing them with the reverse complement, to maximize homology across the inversion region (Figure 3-9) following Whitlock *et al.* (2010). By analyzing the alternative alignment, the new *trnH-psbA* majority-rule consensus tree was no longer as informative and could only form a large polytomy for the whole genus (Figure 3-10). Thus the barcoding of the species within this genus based on only these three regions would be able to identify *A. merriamii*, but would be unable to decipher *A. californica* and *A. humilis* from each other by more than a single nucleotide difference in the *trnH-psbA* region (Table 3-4,
showing results based on both alignments). The final consensus tree incorporating the inverted \textit{trnH-psbA} regions resulted in high bootstrap support for all three species (Figure 3-11).

The Bayesian analysis performed using BEAST and *BEAST also resulted in a clear delineation between the three species (Figure 3-12), supporting the results from the parsimony analysis. The full set of trees was used to construct the DensiTree output, showing the nodes are clearly defined (Figure 3-13). Furthermore the retention index for each gene region partition that informed the combined analysis is extremely robust, ranging from 0.68 for \textit{ITS} to 0.95 in \textit{rbcL} (Figure 3-14).

Using the program GenGIS (**Ghen-GIS**) to map the locations of the populations, and overlay the tree topology and allelic variation, there are clear geographic patterns depicted by the genetic variation (Figure 3-15a), showing that genetic distance and geographic distance are positively correlated among the more widely distributed species and their collection locations (Figure 3-16). For example, the Death Valley populations clearly group together within the \textit{Arctomecon merriamii} branch and for \textit{A. californica} the sites nearest the South Virgin Mountains (Gold Butte) clearly group together, as do the sites within and around Las Vegas (Figure 3-15b). Interestingly, as has been noted based on its unique morphology, the Grand Canyon population (Stash) is clearly distinct at most regions and is a well supported separate branch of \textit{A. californica}. My collaborators and I are describing this as a new variety or sub-species of \textit{A. californica}, and the current data would support this delineation (in prep. Phillips, Simpson and Hodgson 2014).

In comparison to the other two species, the limited geographic range of \textit{Arctomecon humilis} makes the genetic differences between populations less clearly defined, as would be expected (Tables 3-5 and 3-6). There are a limited number of SNPs that differ within the
species, but the different geographic regions clearly have mutations that have arisen independently (Tables 3-7 *A. merriamii*, 3-8 *A. californica*, 3-9 to 3-11 *ITS, ndh-rpl32, rpl32-trnL*). The *A. humilis* branch on individual gene trees lacks structure overall as illustrated by the polytomies in Figure 3-11a. However, this also shows a need for a genetic marker with finer scale resolution. Microsatellite loci are ideal for an analysis at the population level, and should prove particularly useful for *A. humilis*, since the entire geographic range of this species is roughly equivalent to the spatial distribution of the three *A. merriamii* populations in Death Valley.
Figure 3-1. The original analysis majority-rule consensus tree based on six non-coding regions; branches are labeled with bootstrap support values. The $trnH$-$psbA$ inversion is in its original sequence form, overestimating the number of mutations, or sequence differences.
Figure 3-2. Majority-rule consensus individual gene trees based on a parsimony analysis of nrITS run in TNT, with bootstrap support displayed on branches.

nrITS; 710 Characters when aligned with outgroups, 220 informative characters.
Figure 3-3. Majority-rule consensus individual gene trees based on a parsimony analysis of \textit{ndh-rpl32} run in TNT, with bootstrap support displayed on branches.

\textit{rpl32-ndh}; 1285 characters when aligned with outgroups, 181 informative.

A. \textit{merriamii}

A. \textit{humilis}

A. \textit{californica}
Figure 3-4. Majority-rule consensus individual gene tree based on a parsimony analysis of \textit{trnH-psbA} run in TNT, with bootstrap support displayed on branches. This original analysis showed the incorrect grouping of \textit{Arctomecon merriamii} and \textit{A. humilis}, with \textit{Argemone} as well as one of the \textit{Eschscholzia} samples grouping with the rest of \textit{Arctomecon}.

\textit{trnH-psbA}; 507 characters when aligned with outgroups, 84 informative.
Figure 3-5. Majority-rule consensus individual gene trees based on a parsimony analysis of \textit{rbcL} run in TNT, with bootstrap support displayed on branches.

\textit{rbcL}; 1310 characters when aligned with outgroup, 43 informative.
Figure 3-6. Majority-rule consensus individual gene trees based on a parsimony analysis of rpl32-trnL run in TNT, with bootstrap support displayed on branches.

trnL-rpl32; 1179 characters when aligned with outgroups, 170 informative.
Figure 3- 7. Majority-rule consensus individual gene trees based on a parsimony analysis of *matK* run in TNT, with bootstrap support displayed on branches.

*matK*; 853 characters when aligned with outgroups, 73 informative.
Figure 3-8 MAFFT alignment showing the inversion present in the \textit{trnH-psbA} sequences.
Figure 3-9. MAFFT alignment showing the \textit{trnH-psbA} inversion flipped, as it was used in analyses.
Figure 3-10 Majority-rule consensus *trnH-psbA* gene tree with the inversion regions flipped, tree is based on a parsimony analysis run in TNT, with bootstrap support displayed on branches.
Figure 3-11. Majority Rule Consensus tree of six concatenated gene regions (\textit{rbcL}, \textit{matK}, \textit{rpl32-trnL}, \textit{ndh-rpl32}, \textit{trnH-psbA}, and \textit{nrITS}) with Bootstrap support values (\textit{psbA} is inverted)
Figure 3-12. Bayesian tree based on the *BEAST analysis of all gene regions run as separate partitions and then combined to create the species topology.
Figure 3-13. DensiTree output depicting the set of all trees that were retained from the *BEAST analysis of the combined gene regions.
Figure 3-14. Retention Index of the Bayesian trees output by BEAST for each region, with high support for each.
Figure 3-15. The BEAST tree superimposed over the geographic layout of the Mojave Desert, a) showing sites where *Arctomecon* is located, in comparison to the species tree. b) branches colored based on the regions the plants were collected from, to visualize geographic clustering by population.
Figure 3- 16. GenGIS analysis of geographic and phylogenetic correlation \((p < 0.0001)\).
Table 3-1. Non-coding gene regions and PCR cycling parameters for the initial number of samples sequenced at each gene region, and the final number included in the combined analysis.

<table>
<thead>
<tr>
<th>Gene Region</th>
<th>PCR Cycling Parameters</th>
<th>#Characters (informative)</th>
<th>(A.) \textit{humilis}</th>
<th>(A.) \textit{californica}</th>
<th>(A.) \textit{merriamii}</th>
</tr>
</thead>
<tbody>
<tr>
<td>(nr)\textit{ITS}</td>
<td>94°C 2m, (94°C 1m, 55°C 1m, 72°C 1m)x38; 72°C 5m</td>
<td>710 (220)</td>
<td>16</td>
<td>27</td>
<td>28</td>
</tr>
<tr>
<td>(rpl32)-\textit{trnL}</td>
<td>80°C 5m, (95°C 1m, 52°C 1m, 65°C 4m)x28; 65°C 5m</td>
<td>1179 (170)</td>
<td>15</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>(ndh)-\textit{rpl32}</td>
<td>80°C 5m, (95°C 1m, 49°C 1m, 65°C 4m)x30; 65°C 5m</td>
<td>1285 (181)</td>
<td>14</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>(trnH)-\textit{psbA}</td>
<td>95°C 2m, (95°C 30s, 55°C 30s, 72°C 45s)x30; 72°C 5m</td>
<td>507(84)</td>
<td>16</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>(rbcL)</td>
<td>95°C 2m, (94°C 1m, 55°C 30s, 72°C 1m)x34; 72°C 7m</td>
<td>1310 (43)</td>
<td>13</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>(matK)</td>
<td>94°C 2m, (94°C 1m, 48°C 30s, 72°C 1m)x27; 72°C 7m</td>
<td>853 (73)</td>
<td>19</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td># in combined set</td>
<td></td>
<td>5844 (771)</td>
<td>14</td>
<td>19</td>
<td>14</td>
</tr>
</tbody>
</table>
### Table 3-2. Sequences of primers used for PCR amplification and sequencing.

<table>
<thead>
<tr>
<th>Region</th>
<th>Primer Name</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rbcL</td>
<td>1F</td>
<td>ATG TCA CCA CAA ACA GAA AC</td>
<td>Lindquist and Albert 2002</td>
</tr>
<tr>
<td>rbcL</td>
<td>724R</td>
<td>TCG CAT GTA CCT GCA GTA GC</td>
<td>Lindquist and Albert 2002</td>
</tr>
<tr>
<td>rbcL</td>
<td>636F</td>
<td>GCG TTG GAG AGA TCG TTT CT</td>
<td>Lindquist and Albert 2002</td>
</tr>
<tr>
<td>rbcL</td>
<td>1368R</td>
<td>CTT TCC AAA TTT CAC AAG CAG CA</td>
<td>Lindquist and Albert 2002</td>
</tr>
<tr>
<td>matK</td>
<td>matK-390F</td>
<td>CGA TCT ATT CAT TCA ATA TTT C</td>
<td>Cuenoud et al. 2002</td>
</tr>
<tr>
<td>matK</td>
<td>matK-1326R</td>
<td>TCT AGC ACA CGA AAG TCG AAG T</td>
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<td>Blattner and Kadereit 1999</td>
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Table 3-3 Tree statistics from parsimony analyses.

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Taxa: Number of taxa in the nucleotide alignment.
Characters: Number of characters in the nucleotide alignment.
PIC: Number of parsimony informative characters in the nucleotide alignment.
MPTs: Number of equally parsimonious trees found in the tree search.
MPT Length: The length (tree score) of the most parsimonious tree.
Table 3-4. Showing the variable nucleotides of *rbcL matK* and *trnH-psbA* sequences (separated by /), and the non-inverted *trnH-psbA* sequence (separated by //).

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Table 3-5. Variable nucleotides among *Arctomecon humilis* across all gene regions sequenced.

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Table 3- 6. Sequences aligned showing only the variable nucleotides; ITS, matK, ndh-rpl32, rbcL, rpl32-trnL, and trnH-psbA

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Table 3-7. Variable nucleotides among *Arctomecon merriamii* across all gene regions sequenced.

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Table 3. Variable nucleotides among *Arctomecon californica* across all gene regions sequenced.

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Table 3. Variable nucleotides for the *nrITS* amongst the three species.

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Table 3-10. Variable nucleotides for the *ndh-rpl32* amongst the three species.

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Table 3. Variable nucleotides for the rpl32-trnL amongst the three species.

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<thead>
<tr>
<th>Species</th>
<th>Nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acalifornica Airport24</td>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>......................</td>
</tr>
<tr>
<td>Acalifornica Eagle1</td>
<td>......................</td>
</tr>
<tr>
<td>Acalifornica Limel1</td>
<td>.......A</td>
</tr>
<tr>
<td>Acalifornica Lost13</td>
<td>.C...A.T.T.</td>
</tr>
<tr>
<td>Acalifornica Pabco18</td>
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<tr>
<td>Acalifornica Patch46</td>
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<tr>
<td>Acalifornica Rainbow23</td>
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</tr>
<tr>
<td>Acalifornica RedPatch9</td>
<td>...........A.T...</td>
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<tr>
<td>Acalifornica SBurro14</td>
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</tr>
<tr>
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<td>Ahumilis Bee8916</td>
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<tr>
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<td>Amerriamii Castle27</td>
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</tr>
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<td>Amerriamii Horse16</td>
<td>C.CGT.GT...</td>
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<td>C.CGT.GT...</td>
</tr>
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<td>Amerriamii Race22</td>
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</tr>
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<td>C.CGT.GTT...</td>
</tr>
<tr>
<td>Amerriamii Titus29</td>
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</tr>
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<td>C.CGT.GT...</td>
</tr>
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<td>Amerriamii Wash35</td>
<td>C.CGT.GT...</td>
</tr>
</tbody>
</table>

Introduction

*Arctomecon humilis* Coville is a federally listed endangered species and has become one of the top priorities for protection by conservation groups (TNC 2007a). Its narrow range and small populations create a vulnerable predicament, particularly being so close to a rapidly developing city center. The majority of remaining populations are protected in a series of reserves, however between urban development and a lack of further suitable habitat becoming available, the remaining populations of this species are unlikely to expand their current ranges. Mitigation efforts have been made to help preserve the seedbank of this species, but habitats that showed promise for establishing new populations have been destroyed by development (Harper and Nelson 1991, TNC 2007a). Thus determining the population structure and genetic health of the species will be critical to its long-term survival. Effective and sustainable conservation plans for endangered plant species should always take into account the anthropogenic drivers threatening the species, and simultaneously consider the genetic processes at play. Numerous organizations have been trying to develop viable plans to mitigate and manage this species around St. George, UT. These organizations have expressed an interest in determining both the large- and small-scale distribution of genetic variation throughout this largely fragmented landscape. For rare endemic species, genetic variation can be largely shaped by microevolutionary means, and species that are decreasing in population size are especially sensitive to genetic drift and selection pressures across their habitat (Pritchard *et al.* 2000, Manel *et al.* 2010). Processes such as these can influence patterns like isolation-by-distance (Wright 1943) and create genetic substructure across geographic gradients (Manel *et al.* 2003).
A few studies have attempted to describe the genetic variation within *Arctomecon* species (Van Buren and Harper 1996, Allphin *et al.* 1998, Hickerson and Wolf 1998). However, these were unable to offer insights into the genetic variation within populations, or they may not have had sufficient data to interpret the contemporary gene flow between populations. Studies involving conservation genetics of endangered plant species have increasingly turned to highly polymorphic and selectively neutral molecular markers to help identify isolated populations, which can then be treated as unique conservation units (Ansell *et al.* 2010).

The aim of the work described herein was to develop novel microsatellite markers to assess the current state of genetic diversity and gene flow within and among the different populations of *Arctomecon humilis*. This is the first time markers of this type have been developed for this species or genus. Since the complete reserve system has recently been established, determining the levels of genetic variation within this species at this time is extremely important, and has provided insight into the evolutionary processes that are taking place. Microsatellite markers also have higher potential in monitoring conservation status in the future because the same set of primers can be used to assess the allele frequency as a direct comparison. In addition, all markers that were developed for this study were tested for amplification success in the two sister species, *A. merriamii* and *A. californica*. Establishing whether some population groups are more isolated and at risk of inbreeding depression or whether they are experiencing genetic drift will help land managers direct resources to where they are most needed.

**Methods**

*Microsatellite Development*

In the development of useful microsatellite loci, three different methods were utilized: PCR amplification, membrane-enrichment and magnetic bead enrichment. As a first attempt to
develop markers, PCR amplification was conducted. After extraction, DNA was precipitated using 5M NaCl, and 95% ethanol, and then resuspended in 10mM Tris. This DNA was then digested using BSA (100x), NEB buffer1, and the restriction enzyme Sau3AI (NEB 4000 u/µL). Ligation of linkers to the DNA used T4 buffer, T4 ligase (Roche 5 u/µL), and equal parts of linkers NY527, NY528 (100 µM stock). Ligated sections were then selected for using a PCR program, using red buffer (10x stock), BSA, dNTP’s, Taq, and the NY527 linker (10µM stock). Cloning of the PCR product took place with the TOPO vector, and salt solution, added to competent cells. After incubation the competent cells were plated into Petri dishes containing LB media, Kanamycin (0.05 g/L), and x-gal (0.03 g/L). Colonies successfully taking up the inserts were then transferred to 96-well plates containing 100µL LB with Kanamycin (0.05 g/L), and incubated for approximately one hour. PCR screening for microsatellite loci were conducted on this incubation product using; red buffer (10x stock), BSA, dNTP’s, Taq, and different sets of two-, three- and four-nucleotide repeat motif primers (NY529 (GAx15), NY530 (GTx15), NY531 (ATx15), NY532 (GCx15), NY533 (CAAx10), NY534 (CATAx10), NY535 (ATTx10), NY537(GCCx10)), each serving as reverse or forward primers with the compliment M13 primers (NY755 or NY756), respectively. PCR products were run out on agarose gels to check for distinct bands of the products. When products of proper size were obtained, they followed the same methodology for sequencing and primer design as explained with the enrichment techniques below.

The other two enrichment techniques, membrane and magnetic bead, followed the same basic protocol to derive the digested DNA up to the point of hybridization. Microsatellite-enriched genomic libraries were first constructed using DNA extracted from leaf samples of the focal species, *A. humilis*. Genomic DNA was isolated from leaf tissue collected during the fall of 2009, using the DNeasy Plant Mini kit (Qiagen). The DNA was quantified with the Nanodrop
1000 (Thermoscientific). Approximately 100-200ng of DNA was ethanol precipitated and then digested with Sau3A1 for 18 hours at 37°C, followed by 20 minutes at 65°C (New England BioLabs) in a 100µL reaction according to the manufacturers recommended protocol. The resulting digestion was run through a dephosphorylation step using Shrimp Alkaline Phosphatase (Promega 1unit/µL) for 1 hour at 37°C, then deactivated at 65°C for 15 minutes. Linkers A and B (Moraga et al. 1998) were assembled (95°C for 10 min, cooled to 10°C over 4.25 h), and were then ligated to the digested genomic DNA for 16 h in a 115µL reaction according to the manufacturer’s protocol. At this point, the DNA fragments used for the enrichment process were either selected for using the membrane enrichment method, or the magnetic bead enrichment method.

Membrane preparation (Roche Nylon Cat# 11417240), hybridization, washing, and elution followed the methods of Edwards et al. (1996) with slight modifications. Prior to membrane hybridization the DNA elutant was amplified with Linker A (20 cycles: 95°C 2 min; 95°C 30 s, 59°C 30 s, 72°C 30 s; 72°C 10 min) in a 100µL volume. The amplicon was then rehybridized, eluted, and reamplified by repeating the same protocol as above, in a 25µL volume (Little, in preparation).

Magnetic Dynabead enrichment followed the protocol of Glenn and Schable (2005). The hybridization solutions contained a variety of biotin labeled oligonucleotides consisting of repeats: CA(10), AT(12), GTT(8), AAG(8), CAG(8), GTG(8), GAT(8), TAG(8), and TCA(8). Linker ligated DNA fragments were first hybridized to the biotin labeled oligonucleotides. The hybridized fragments were then hybridized to the streptavidin beads (Invitrogen Cat# 112-05D). Hybridized beads were washed and the resulting enriched DNA was eluted in 200 µl of TLE buffer. This DNA was then run through an ethanol precipitation and resuspended in 25µl TLE.
Enriched fragments were amplified by PCR with Linker A as the amplification primer (Glenn and Schable 2005).

**Cloning and Sequencing**

The final microsatellite-enriched amplicons in both the membrane and magnetic bead enrichment protocols were cloned with the pCR 2.1 vector into One Shot Top10 Chemically Competent Cells (Invitrogen), following the manufacturer’s protocol. Transformation of the cells followed the manufacturer protocol. Culture plates were prepared with kanamycin and X-Gal, for colony screening. White colonies were used to inoculate 100µl tubes of LB broth (Sigma), also containing kanamycin (50μg/µl) and incubated for approximately one hour at 37°C. Colonies were selected through PCR amplification with M13 forward and reverse primers in 15µl reactions (95°C 10 min.; 95°C 30 sec., 55°C 30 sec., 72°C 1 min. for 35 cycles; 72°C 10 min.) reactions containing the following reagents: 1.5 µL buffer, 0.2 mM dNTPs, 0.025 µg/µL BSA, 3 µL Q solution (Qiagen), 0.4 µM M13 primers, 0.1 units Taq (Qiagen), and 1 µL bacterial culture. PCR products were run on a 1% agarose gel alongside a 1 Kb ladder (New England Biolabs) and products containing inserts in the 500-800 bp range were sent off-site for sequencing.

PCR products were cleaned with ExoSAP-IT and sequenced with M13 primers on an Applied Biosystems 3730 sequencer (High-Throughput Genomics Unit, University of Washington, Seattle, WA). Resulting sequences were edited and assembled using Sequencher 4.10.1 (Gene Codes). Contigs were then exported as Fasta files and run through read2Marker (Fukuoka et al. 2005) and an online program WebSat (Martins et al. 2009) in order to identify microsatellite repeat motifs and design suitable primers sets.
454-sequencing

In addition to the two hybridization techniques, 454-sequencing of the enriched and total genomic DNA was performed by the United States Geological Survey (USGS 2006), at the Leetown Science Center in West Virginia. The Roche 454-sequencing rapid library preparation method was used to generate and align sequences (454 Life Sciences Corp. 2010). For this method two sample tubes of high-quality genomic DNA and one tube of magnetic bead-enriched DNA were sent to the USGS lab. DNA enriched for hybridization had already been digested and selected for fragments containing repeats ranging from roughly 400-800bp. Genomic DNA was fragmented by Nebulization, hybridized to magnetic beads, and according to their protocol was supposed to select for fragments between 500-1250bp (454 Life Sciences Corp. 2010). Average length of the 249,405 fragments was actually 370bp. As fragments overlap they can be matched and aligned together to form longer sequences, however many fragments will also be left unmatched. All 454-sequences were first run through MsatCommander (2008) in order to identify fragments containing repeat motifs. All fragments containing repeats were then run through WebSat (2009) in order to design primers. Fragment sequences were also run with read2Marker as a crude comparison with the two hybridization methods.

Primer testing and Genotyping

Microsatellite primers from all three methods were evaluated for functionality among samples of Arctomecon humilis representing its entire geographic range. The microsatellite primers were additionally tested for cross-reactivity among the other two members of the genus, A. californica and A. merriamii.

Primers were initially tested for PCR amplification using a temperature gradient (~47-57°C), and an increased number of cycles. Primer pairs that successfully amplify products with
these parameters were then optimized for temperature and number of cycles, for each species. General test PCR reactions were run at half volume (12.5µL) and contained the following reagents: 4.65 µL H$_2$O, 1.25 µL buffer, 1.25 µL dNTPs (0.2 mM), 1.25 µL BSA (0.025 µg/µL), 2.5 µL Q solution (Qiagen), 0.5 µL forward primer, 0.5 µL reverse primer, 0.1 units Taq (Qiagen), and 0.5 µL DNA. Cycling parameters were run at 95ºC 2 min.; 95ºC 30 sec., 51-55ºC (depending on the locus) 30 sec., 64ºC  45 sec. for 29-32 cycles (depending on locus); 65ºC 5min. Once suitable polymorphic microsatellite loci were identified (following criteria in Selkoe and Toonen 2006) the remaining samples were run through the same optimized procedure and sequenced for population genetic analysis.

Once the general PCR parameters were confirmed to amplify samples, they were tested for amplification with dye primers that are detected by the CEQ Fragment Analysis System (Beckman Coulter). PCR reagents are similar to above, however the forward primer is reduced to half volume and a dye primer is added at a volume equal to the reverse primer volume (cycling parameters remain the same). All PCR products are run out on an agarose gel, stained with ethidium bromide and imaged under UV lamp. All samples were then prepared for genotype fragment analysis.

**Genotyping**

The Beckman Coulter CEQ detects the nucleotide length of DNA fragments. PCR products labeled with one of three M13 dye tails were loaded into a formamide solution containing 0.5µL 400-bp size standard. According to the manufacturer’s recommendation, product volumes are loaded based on dye type, as follows: R-tail (blue) 0.5 µL, -40-tail (green) 2.0 µL, and -20-tail (black) 4.0 µL. PCR products with differing dye labels can be pooled together into the same reaction well on the injection plate, the total volume of the reaction well is then brought up to a total of 40 µL with formamide. Each fragment peak is verified manually by
checking the size calibration data; any peak with a standard deviation greater than 0.5 nt is rejected. The fragment data produced by the CEQ was then downloaded and run through GeneMarker 1.95 (SoftGenetics) for further verification of peak size and fragment length. Initial determinations of peak calls by GeneMarker were verified manually for each locus, these peak calls were then used to create allele ‘bins’ for future runs of each locus (Figure 4-1 for example of peak calls, and allele binning). The number of overall alleles per locus is determined in GeneMarker, and an analysis panel is created for each locus. Panels are then used for subsequent CEQ runs, and modified as needed (i.e. new alleles are identified and additional bins included). To ensure that fragment lengths were being assigned consistently by both the CEQ and GeneMarker software, anywhere from 3-10% of the preliminary samples per locus were run a second time to verify fragment length.

Data Analysis

Bacterial colonies containing inserted target DNA were run through PCR, electrophoresis gels and imaged in order to verify the transgenes were amplified. The images were then imported into Image-J 1.46 (Rasband 1997) where DNA bands could be measured to ensure properly sized products were sent for sequencing (Fig 4-2). The genotype data for all suitable microsatellite loci resulting from the preliminary CEQ runs and determined through GeneMarker were uploaded into Excel (Microsoft Office 2007). GenAlEx (Genetic Analysis in Excel) is an Excel ‘Add-in’ that provides a wide range of population genetic analyses, as well as numerous data exporting options (Peakall and Smouse 2006). The number of alleles per microsatellite locus, observed ($H_o$) and expected heterozygosity ($H_e$), conformance to Hardy-Weinberg equilibrium (HWE) expectations, and population genotypic disequilibrium using pairwise tests were run using GenAlEx v. 6.41 (Peakall and Smouse 2006). An additional data set consisting of
Results

Development and Testing of Microsatellite Markers

Efficiency of the three microsatellite enrichment techniques applied here varied widely. The PCR-enrichment was initially appealing due to the need for fewer materials and lower monetary expense. However, this method proved to be inefficient and time consuming in terms of verification of results and troubleshooting. While no loci were used in this study, it did provide a basic procedure to follow while carrying out both the membrane- and the magnetic-bead-enrichment techniques, as they all contain similar steps. The efficiency, and thus productivity and progress of each method were determined by the success of fragment amplification after bacterial transformation.

The PCR-enrichment technique did not provide any variable markers for this study. Of the four 96-well plates that were set up with transformed bacterial colonies, and subsequent PCR amplification, only 121 had inserts. Ninety-six of these samples were sequenced, but only seven contained repeat regions as detected by read2Marker. Two primer pairs were designed and ordered, however they failed to amplify the target region (Table 4-1). The membrane-enrichment method showed improvement over the PCR technique, however efficiency was still low, and the cost of sequencing plates made this method impractical. Over the course of a few months, fourteen 96-well plates were used for colony culture, and PCR amplification. Upwards of 1180 wells contained bacteria that had taken up the target DNA, and products were measured for target size (500-800bp). Four hundred-three were sent to be sequenced, 40 of those had repeat regions (9.3% efficiency), with 18 having primers designed. Upon testing these primer sets, three successfully amplified, but only one was variable.
The magnetic-bead-enrichment method proved to be far more efficient and provided markers that were variable at a more consistent rate. Of the nine 96-well plates containing screened bacterial colonies and subsequent PCR amplification, 573 had inserts of appropriate size. Ninety-six of these were sent for sequencing, 37 had repeat regions (38.5%), 16 had primers designed, 12 of them successfully amplified DNA fragments. Ten of these primer sets were used to construct the preliminary genotyping database. The number of alleles per locus ranges from 2 to 20 alleles.

The magnetic bead hybridization was not only more successful and efficient at selecting repeat regions, but also had a higher average number of repeats per motif as compared to the membrane hybridization technique (Figure 4-3). The higher number of repeats per locus increases the likelihood of variability at a given locus due to the increased chance of slippage during DNA replication.

The final method that was utilized to identify and develop microsatellite markers was next generation technology, 454-sequencing. This method produced 6,314 DNA fragments of *Arctomecon merriamii* sequences, and 49,781 fragments of *A. humilis* DNA. In total, this includes over 36 million base pairs of data when considering both the matched and unmatched fragments. However, it should be noted that the enriched DNA contained more duplicate fragments than the genomic DNA. This is likely due to the natural selection and replication of certain fragments during the uptake and incubation steps in the enrichment technique (Glenn and Schabel 2005), whereas the genomic DNA lacked a pre-selection step. Overall, the 454-fragment data provided an enormous amount of potential microsatellite markers as identified by read2Marker (>3500 primer sets in all). Since this method was last to be tried, and was somewhat unexpected, time constraints and funding allowed only a fraction of a percent of these primer sets to be ordered and tested. It should be noted that while far more fragments were
obtained from the previously enriched DNA, these fragments did not have a higher efficiency or percentage of repeat regions when compared to the genomic DNA (Table 4-2). This may be due to the fact that the enriched DNA had been developed using a limited set of oligonucleotide repeat motifs, whereas the genomic DNA still had all the possible repeat motifs present in the original genome. The read2Marker program was able to identify many duplicate sequences in the enriched DNA, but that number of duplicates is not likely to catch the true number of duplicate fragments, or partial fragments in the matched alignments. Although the 454-sequencing did not obtain nearly as many fragments from the genomic DNA compared to the enriched DNA, this may be due to the individual sequencing run, as the results from one sample may swamp out the other samples in the same run (personal communication, Johnson 2011).

While all primer sets could not be tested, the vast number of fragments did allow the use of extremely high search parameters in MSATCOMMANDER (>15 tri- and tetra- nucleotide repeats) that increased the likelihood of variable markers and complimented the markers that were developed using the enrichment techniques. The dataset of microsatellite containing sequences that was output by MSATCOMMANDER was then run through WebSat, in order to design primers. Nineteen primer sets were ordered, twelve amplified in at least one species, and eight amplified in all three species. Seven of the 454-markers were tested for allele variation; all seven were found to be variable in at least one species. However, one (454seq12) had products that were above 400bp, and the use of this locus was discontinued.

Three loci in particular were found to be amplifying more efficiently than the others and were variable. Additionally, two markers (454seq04 and 454seq18) were variable among individuals of all three species, but always possessed only a single allele peak in each sample, never any heterozygotes. This is highly unexpected for a marker that is variable within each population, as allele frequencies among individuals of all the other markers exhibited individuals
that were heterozygous at each respective locus. Upon further investigation and the results of a GenBank Blast of nucleotide sequences, it was discovered that these two regions matched mitochondrial DNA of other plant species. In addition, since mitochondria have different mutation rates and a separate evolutionary lineage when compared to the nuDNA, these two loci were correlated with each other. In all subsequent analyses these two loci were treated as haploids, and individuals were identified by either a single haplotype or a homozygous diploid, depending on the program parameters (Pritchard 2010, Peakall and Smouse 2009). Genotyping was still performed for all three species. However, with the limited number of markers amplifying in all three species, the majority of the population genetic analyses were run in more depth on *Arctomecon humilis*.

Originally, the goal was to use only microsatellite loci that successfully amplified in all three species of *Arctomecon* consistently and with a similar level of variability. However, this strategy had to be abandoned, as cross amplification was less successful than expected. The lack of cross amplification can result from the amount of genetic distance between the species due to changes in the nucleotide sequence and thus primer binding success. Additionally, when amplification did occur, variability in the other two species was often highly reduced or fixed at a single allele size. If loci were found to be monomorphic during the preliminary analysis or alleles were too large, these loci markers were not pursued for further development (i.e. locus 1E7 amplified well, but product size was larger than the 400bp size standard used in the CEQ).

Among the 16 loci that were variable and amplifying well within *Arctomecon humilis* there are a total of 154 alleles (Table 4-3). Three primer-pairs that were part of the preliminary data set have been discarded, and were not used in the *A. humilis* population study either due to unclear fragment peaks (3G4), or because the fragments were too large and could not be accurately genotyped (1E7 and 454seq12). The cross-application of the primers within the other
two species has not been as successful as originally expected. However, the lack of cross-application does support the robust species delineation seen in the other phylogenetic information (Chapter 3). Of the 16 variable loci amplifying well in *A. humilis*, only 9 amplified in *A. merriamii* while 13 are amplifying in *A. californica* (Table 4-3). Amongst the loci that are amplifying in *A. merriamii*, five are fixed at a single allele, thus only four loci were included in the larger data set. Among the 13 loci that are amplifying in *A. californica*, four are fixed, and four have had difficulty amplifying samples with clear peaks, leaving five variable loci to pursue.

Since *Arctomecon humilis* was the focal species of this study, its genomic DNA was used for initial development and the amplification success reflects this situation. However, while the success (or lack thereof) for microsatellite amplification within these sister species cannot be directly translated to the correct genetic relationship within the genus, it is interesting to note that these results agree with the phylogentic data and also support *A. humilis* and *A. californica* as sister species, with *A. merriamii* being more ancestral (i.e. greater amplification success in *A. californica*, less in *A. merriamii*).
Figure 4-1. Examples of Peak determination in GeneMarker analysis software. Each of these panels has dozens of samples overlaid on one another in order to determine many alleles at once. After the initial creation of the panel, large sets of sequences can be run through and when a peak falls within this range it will be labeled. This shows three trinucleotide loci.
Figure 4-2. Example of gel image used to estimate size of target DNA inserts, those that should contain microsatellite motifs.
Figure 4-3. Comparing the number of repeats per motif region based on the two hybridization techniques.
Table 4-1. A comparison of efficiency among the microsatellite development techniques, based on repeat regions identified and the number of primers successfully amplifying products in PCR.

<table>
<thead>
<tr>
<th>Method</th>
<th># of colonies transformed (fragments sequenced)</th>
<th># fragments with repeat region (% efficiency)</th>
<th># with primers designed</th>
<th>successfully amplified</th>
<th># with variable loci (<em>A. humilis</em>)</th>
<th>Efficiency of primers, from those ordered</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR-enrichment</td>
<td>121 (96)</td>
<td>7 (7.3%)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Membrane-enrichment</td>
<td>1180 (403)</td>
<td>40 (9.3%)</td>
<td>18</td>
<td>3</td>
<td>1</td>
<td>5.6%</td>
</tr>
<tr>
<td>Magnetic Bead-enrichment</td>
<td>573 (96)</td>
<td>37 (38.5%)</td>
<td>16</td>
<td>12</td>
<td>10</td>
<td>62.5%</td>
</tr>
</tbody>
</table>
Table 4-2. Comparison of efficiency between genomic and enriched DNA in 454-sequencing reads.

<table>
<thead>
<tr>
<th>454-sequencing</th>
<th>Total # Fragments</th>
<th># of Fragments with Repeats</th>
<th>% Efficiency</th>
<th># of Primer sets designed</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. merriami</em>; Genomic DNA</td>
<td>794 matched (491,592bp)</td>
<td>104</td>
<td>13.1%</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>5520 unmatched (1,931,536bp)</td>
<td>651</td>
<td>11.8%</td>
<td>332</td>
</tr>
<tr>
<td><em>A. humilis</em>; Genomic DNA</td>
<td>176 matched (94,219bp)</td>
<td>25</td>
<td>14.2%</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>1319 unmatched (433,440bp)</td>
<td>147</td>
<td>11.1%</td>
<td>70</td>
</tr>
<tr>
<td><em>A. humilis</em>; Enriched DNA</td>
<td>20,108 matched (9,329,962bp) (26 duplicate fragments)</td>
<td>1663</td>
<td>8.3%</td>
<td>1151</td>
</tr>
<tr>
<td></td>
<td>28,178 unmatched (10,742,650bp) (2368 duplicate fragments)</td>
<td>3146</td>
<td>12.2%</td>
<td>1920</td>
</tr>
</tbody>
</table>
Table 4-3. Loci that successfully amplified in *Arctomecon humilis*, and subsequent preliminary tests in *A. californica* and *A. merriamii*. Locus p2E3 was developed with membrane enrichment, 3A4-3G4 were developed using the bead enrichment technique, the remaining were developed using 454-sequencing. NA=No Amplification, N=sample size during preliminary analysis, He=Expected heterozygosity. * = discarded markers (due to large product size, unclear peak calls, or monomorphism).

<table>
<thead>
<tr>
<th>Locus</th>
<th><em>A. humilis</em></th>
<th><em>A. californica</em></th>
<th><em>A. merriamii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Allele size range</td>
<td># of alleles N</td>
<td>He</td>
</tr>
<tr>
<td>p2E3</td>
<td>247-285</td>
<td>12</td>
<td>47</td>
</tr>
<tr>
<td>3A4</td>
<td>344-365</td>
<td>7</td>
<td>36</td>
</tr>
<tr>
<td>3B12</td>
<td>345-348</td>
<td>2</td>
<td>66</td>
</tr>
<tr>
<td>9H1</td>
<td>200-215</td>
<td>6</td>
<td>63</td>
</tr>
<tr>
<td>3G6</td>
<td>213-225</td>
<td>5</td>
<td>48</td>
</tr>
<tr>
<td>p3E4</td>
<td>373-385</td>
<td>5</td>
<td>41</td>
</tr>
<tr>
<td>1A12</td>
<td>291-303</td>
<td>5</td>
<td>53</td>
</tr>
<tr>
<td>3G8</td>
<td>351-409</td>
<td>14</td>
<td>64</td>
</tr>
<tr>
<td>9H3</td>
<td>170-173</td>
<td>2</td>
<td>72</td>
</tr>
<tr>
<td>*1E7</td>
<td>408-418</td>
<td>5</td>
<td>36</td>
</tr>
<tr>
<td>*3G4</td>
<td>182-192</td>
<td>5</td>
<td>55</td>
</tr>
<tr>
<td>454seq01</td>
<td>207-258</td>
<td>17</td>
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<tr>
<td>454seq02</td>
<td>292-316</td>
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<td>63</td>
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<tr>
<td>454seq03</td>
<td>178-217</td>
<td>12</td>
<td>77</td>
</tr>
<tr>
<td>454seq04</td>
<td>283-318</td>
<td>8</td>
<td>74</td>
</tr>
<tr>
<td>454seq05</td>
<td>184-238</td>
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<td>70</td>
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<td>454seq11</td>
<td>296-389</td>
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<td>51</td>
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<tr>
<td>*454seq12</td>
<td>365-414</td>
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<td>24</td>
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<tr>
<td>454seq18</td>
<td>334-359</td>
<td>6</td>
<td>23</td>
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</table>
Chapter 5 Population Genetics of *Arctomecon humilis*

**Introduction**

This study builds upon previous genetic assessments of *Arctomecon humilis*, but is aimed at describing fine scale evolutionary processes within and between the populations across the rapidly developing landscape. Microsatellite markers are known to mutate at a more rapid pace than alternative genetic markers. By incorporating them into the conservation strategies being implemented to protect this species, a more informed and data supported management plan will be able to be put in place. In addition to determining the overall genetic variation that exists for the species we can also discover which populations are becoming more isolated and experiencing genetic drift, those that are at risk of inbreeding depression, as well as which localities may be more genetically diverse and acting as genetic source populations or potential corridors among the soil ‘islands’ that the populations of this species inhabits. In addition to examining genetic structure at these fine spatial scales, the hypothesis that established reserves are effectively protecting the genetic diversity of this species can also be tested. This work can be compared to the previous genetic study and determine how effective the microsatellite markers are at capturing fine scale processes within the populations.

All of the known populations of *Arctomecon humilis* have been included in this study, and at some locations the entire effective population was sampled. Comparisons between populations or genetic clusters that are within and outside of the reserve system should be able to determine whether the greatest amount of genetic diversity is currently being protected. The microsatellite markers can also be used to determine populations with private alleles, and thus aid in setting up protected corridors between the isolated populations. A study comparing the soils at each location where these collections were made, found that the overall variation in soils was almost as high within a site, as it is between sites, and that each site had a similar range of
the tested variables, making the different sites fairly consistent across the region (Harper and Nelson 1991). In order to test the hypotheses of variation among age classes and soil types, AMOVA tests were run. Genetic analysis in combination with demographic and community information elucidates the population structure of this species.

Methods

In addition to visiting the known populations, attempts were made to identify new pockets of plants by searching potential habitat for undocumented populations. I observed numerous patches of soil that would seem promising for additional population establishment, or where local populations may have been extirpated. In the end, tissue sampling was conducted for plants collected from thirteen localities (Figure 5-1). The original plan was to sample from 30 individuals at each site across the available range, however multiple collection localities were significantly smaller than others and had fewer than 30 individuals. Once ‘population’ groups were identified and sampled, DNA extractions and genotyping was conducted (Chapters 2 and 4).

Analysis of Population Structure

All of the allele calls for each individual were uploaded into a FileMaker database along with digital photograph vouchers, age class information, and site characteristics. The complete dataset, including all three Arctomecon species was exported to Excel. The program GenAlEx, and Excel add-in, was used first to convert and export data files to other analysis programs. All members of the genus were grouped at the population and species level, and analyses including classical summary statistics, as well as Bayesian approaches (Excoffier and Heckel 2006) were performed in order to understand genetic diversity at the different levels of biological organization and spatial distribution (Manel et al. 2007). The program BAPS (Bayesian
Analysis of Population Structure v. 5.3, was first run in order to infer the number of genetic population clusters (K), and to determine whether certain populations exhibited limited gene flow (Corander et al. 2009). In addition to BAPS, the program Structure was also used to estimate K and assign individuals to distinct populations (Pritchard et al. 2000).

The two population clustering algorithms used by BAPS and Structure partition the individual multilocus genotypes over a set of clusters, but they still involve a stochastic simulation, which can cause replicate cluster analyses of the same data to produce several distinct solutions for the estimated cluster membership (Jakobsson and Rosenberg 2007). Multiple series of Structure iterations were run and then were combined by uploading them into STRUCTURE HARVESTER v0.6.92, a web-based program that allows for greater visualization options of the Structure output and implements the Evanno method of clustering individuals in order to determine the best value of K identified in Structure (Earl and vonHoldt 2012, Evanno et al. 2005). The Evanno method as implemented in STRUCTURE HARVESTER will detect the number of populations that best fit the dataset. The individual and population files for each value of K are output from STRUCTURE HARVESTER and were then directly input to CLUMPP v 1.1.2 (Clustering Matching and Permutation Program) (Jakobsson and Rosenberg 2009). Within CLUMPP, the input files consisting of multiple runs of estimated cluster membership coefficient matrices are permuted so that all replicates have as close a match as possible, allowing ‘genuine multimodality’ to be detected (Jakobsson and Rosenberg 2007). As the value of K increases, the permutations take exponentially longer, thus for K<7 the ‘FullSearch’ algorithm was employed, for K between 7 and 14, the ‘Greedy’ algorithm was used, and for K>14, the ‘LargeKGreedy’ algorithm was implemented in order to speed up the process (Jakobsson and Rosenberg 2009). Finally, the CLUMPP outfile was uploaded into Distruct v
1.1 in order to have greater control of visualizing and labeling the individual estimated membership with the K clusters (Rosenberg 2004).

The values of K that were identified as best fitting the data were subsequently used to define the number of genetic clusters or ‘populations’ in GenAlEx, where additional genetic analyses were then performed. These genetic analyses included the level of genetic variation for each successfully amplifying microsatellite locus, allele frequencies, percent polymorphic loci (P), number of alleles per locus (A), mean observed heterozygosity (H_o), and the expected heterozygosity under Hardy-Weinberg expectations (H_e). Population dynamics were assessed through Analysis of Molecular Variance (AMOVA) determining Wright’s fixation index, F, which ascertains the level of heterozygosity within populations and whether they are within Hardy-Weinberg equilibrium (Wright 1951). In addition, since F_{ST} is known to underestimate the level of genetic variation among highly polymorphic markers (such as microsatellites), particularly when within-population variance is nearly as high as the total variance, three alternative genetic differentiation measures were calculated, G’_{STest} (Nei 1983), theta’_{ST} (θ'_{ST}) (Weir and Cockerham 1984), and D_{STest} (Jost 2008). The modifications of these F_{ST}–analogues (φ_{ST}) create a standardized statistic by dividing the found φ_{ST} by the maximum possible φ_{ST} value when given the present within-population data (Meirmans 2006, Hedrick 2005).

In order to incorporate spatial data, comparisons of genetic distance to geographic distance between populations were determined using Mantel tests (Mantel 1967). Total genetic diversity was then parsed into genetic variation of both within (H_s) and among population (D_{ST}) across the broader geographical regions, again using an AMOVA analysis (Peakall and Smouse 2009). This research informs conservation measures on a population level within the reserve system.
Results

Genetic Cluster Assignment

The data set containing the three *Arctomecon* species was run in Structure, exploring values of K from 1 (three species comprise the same genetic cluster) to 38 (each collection site is genetically unique). While the genotype data for both *A. californica* and *A. merriamii* was highly lacking in comparison to *A. humilis*, missing data is permitted in Structure, but the analysis was run using only the loci that the three species shared in common. According to the Evanno table, the 3 best estimates of K when the three species are included are 25, 17, and 28 (Figure 5-2). Even with limited data the cluster assignment supports the results from the phylogenetic analysis, by first clustering the genotypes by species, then by the populations within each species. Additionally, populations are largely grouped by geography as well as genetic differentiation as shown in the Q plots for the highest values of K (Figure 5-3).

*Arctomecon merriamii* has the least amount of data present in the analysis, and correspondingly the genetic structure doesn’t change considerably above K=6. The higher number of alleles and population structure in *A. humilis* is largely driving the value of K higher in the combined analysis, although *A. californica* shows significant population structure as well. The remainder of this chapter will focus on the complete set of *A. humilis* genotype data and population analysis, with references to the other two species in relation to this focal species.

Genetic Clustering of *Arctomecon humilis* individuals

The *Arctomecon humilis* genotype data was used in the genetic cluster analysis first performed using BAPS to assign individuals to populations, without any predefined population or collection site information. Running multiple iterations with varying starting points of K (1-20), BAPS identified 12 genetic clusters as the number of populations that best fit the data. The optimal partition for 12 clusters was Log(marginal likelihood) of -11402.39, however the
Log(ml) essentially levels off at 10 clusters (i.e. Log(ml)=−11427 at K10, Log(ml)=−11407 at K11, and Log(ml)=−11405 at K13). The number of collection sites is thirteen, and at K12 each collection site is clearly clustering independently, other than the collections made on the north and south sides of Boomer Hill west of Santa Clara. By running BAPS multiple times with a single fixed value of K, it was found that 4.9% of the individuals were being assigned to different clusters, showing limited admixture between those groups. The same data set was then run through Structure with values of K starting at 1 and ending at 18 with 12 iterations for each value of K. The result of all the Structure iterations was then uploaded to Structure Harvester and CLUMPP in order to synthesize all of the different iterations. According to the Evanno method run in Structure Harvester, the number of K groups that were detected and that best fit the data was 10 (Figure 5-4). According to the 10 genetic clusters, the collection sites were largely maintained, with the sites around Boomer Hill appearing to form a single population (Boomer Hill, Gnarly, and South Butte), and significant admixture between Sun River and Bloomington (Figure 5-5). It should be noted that the population structure detected at K=4, does show a greater amount of admixture between the different regions around St. George. For example, the westernmost populations, including Red Bluff are clustered together, the populations around Price Hills create a cluster, the two sites furthest north and east (Shinob Kibe and Warner Ridge) are clustered, as are the two furthest southeast (Beehive Dome and White Dome). These two sets of genetic clusters were used to inform the GenAlEx population genetic analysis using K=10 as the populations and K=4 as regions groups.

*Population Genetics of Arctomecon humilis*

In relation to the regional clusters, it should be noted that gene sequence data that has been obtained, as well as the data from previous genetic studies (Van Buren and Harper 1996, Allphin *et al.* 1998, Hickerson and Wolf 1998) and the phylogenetic data from this study, did
find that the westernmost populations of *Arctomecon humilis* were genetically unique in comparison to the other populations (Figure 5-6). This does not necessarily mean they are more variable (although at some loci they are), but rather there are genetic alleles that show up more often in that region, which are not found as commonly in the other populations. This may suggest that these populations have been separated for a longer period of time from, or do not exchange genetic material as often with, the populations near the center, and east of St. George. This study utilizing microsatellite markers confirms that result, but also detects genetic differentiation on a finer scale (allele maps shown in Appendix B, allele frequencies per locus in Appendix C).

The number of individuals per population included in the study ranged from 26 at Price Hills to 49 at Boomer Hill. The deviations from the goal of 30 individuals per site were due to some samples not amplifying consistently, or the grouping of the western populations. Of the sixteen markers that were used for this study, the number of alleles per locus ranged from 2 to 23. Additionally the two mitochondrial loci (454seq04 and 454seq18) were recoded in the overall data set as a single haplotype, since they were found to be linked, as suggested by Pritchard (2010). This resulted in 30 unique haplotypes for these two markers. One of the more widely reported statistics in population genetics is Wright’s F-statistic (1951), which can range from 0.0 (no differentiation) to 1.0 (complete differentiation – i.e. subpopulations fixed for different alleles). Although $F_{ST}$ has a theoretical range of 0-1.0, the observed maximum is usually much lower than 1.0, particularly for highly variable loci such as microsatellites. According to Wright (1978) when considering variable allozyme loci; 0-0.05 indicated little genetic variation, 0.05 to 0.15 moderate, 0.15-0.25 great genetic variation and $F_{ST}$ above 0.25 very great genetic variation. For these populations the $F_{ST}$ ranges from a low of 0.07 at Price Hills, to a high of 0.34 at Boomer Hill (Table 5-1). The greater diversity at Boomer Hill is likely
to reflect the larger geographic area that the samples were taken from and combined. The overall $F_{ST}$ for the species is 0.15, indicating limited to moderate differentiation. However, the traditional calculation of $F_{ST}$ is not able to capture the true amount of variation when dealing with hyper-variable markers such as microsatellites (Hedrick 1999, 2005), instead the analogues to $F_{ST}$ are reported below. Finally, using the same data that produced Table 5-1, Shannon Diversity Index takes the frequencies of alleles and population sizes, and provides a convenient measure of diversity among populations (Figure 5-7).

In addition, the $F_{ST}$ values reflect the Hardy-Weinberg Equilibrium Chi-sq analysis that was performed for each locus done by population, in that the populations with lower $F_{ST}$ values had fewer loci showing significance (i.e. Price Hills, 1 of 14 loci was statistically significant), and thus could not reject the null hypothesis of no departure from random mating. Those with a high $F_{ST}$ value, also showed a greater number of loci that were statistically significant (i.e. Boomer Hill, 11 of 14 loci were statistically significant), or a rejection of the null hypothesis, and a departure from the random mating expectations of the Hardy-Weinberg Equilibrium.

In order to determine the hierarchical partitioning behind the genetic differentiation, two separate Analysis of Molecular Variance (AMOVA) were performed, first with input of the individuals ($n=341$) and populations ($K=10$) (Figure 5-8a), then adding the regions ($K=4$) (Figure 5-8b). In addition to the AMOVA being run on the observed data, the complete data set was shuffled 999 times with AMOVA being calculated for each shuffle. The observed data differs significantly from the randomized data, thus supporting the genetic difference among the populations ($\Phi_{PT}=0.298$, $P=0.001$, Figure 5-9).

As was seen in the phylogenetic analysis, and most of the population genetics, the relationship between genetic distance and geographic distance was evident. This isolation-by-distance can be tested with a Mantel test based on the genotyping data and the GPS waypoint
information. The results of the Mantel test (Figure 5-10) confirms the statistically significant positive relationship between genetic distance and geographic distance (R=0.473, p=0.01).

Within GenAlEx, a genotypic distance measure is used for the AMOVA tests. The genotypic distances are summed across all loci, this method is able to suppress intra-individual variation and is therefore able to produce $\Phi_{PT}$ (Excoffier et al. 1992), which is more appropriate for microsatellite data but is also ideal for comparing between codominant and haploid data. In addition to this value, one can calculate $F_{ST}$-analogues when dealing with highly polymorphic markers. Using a combination of the programs SMOGD, Recode, and Fstat, the $\varphi_{ST}$ values of $G'_{ST}$ (Nei 1983), $\theta'_{ST}$ ($\theta'_{ST}$) (Weir and Cockerham 1984), and $D'_{STest}$ (Jost 2008) were found. The values of 0.427 $G'_{ST}$ and 0.45 $\theta'_{ST}$ show a much great amount of differentiation that was not captured by the $F_{ST}$. Jost’s D was more conservative ($D'_{STest}=0.3$), and is similar to the AMOVA results. Additionally, using the results of D to calculate the harmonic mean of $D_{est}$ across loci, the pairwise distances of population difference can produce a more accurate PCA that reflects the Structure results more closely (Figure 5-11).

**Discussion**

**Conclusion and Recommendations**

According to the phylogenetic analysis, the microsatellite amplification data, and the number of fixed alleles or reduced variation among these markers, it is clear that the divergence between *Arctomecon humilis*, *A. californica* and *A. merriamii* is not a recent event. Although the three species share some alleles amongst these markers, the different mutation patterns and the high degree of alleles that are not in common support a more distant differentiation. It is interesting to see that the mitochondrial markers were amplifying consistently in all three species, suggesting a different evolutionary pattern than the other markers, although the region that marker 454seq04 is amplifying in *A. merriamii* is unlike than the other two species. This
data does support the status of variety or sub-species for the Grand Canyon population, but it would be best to obtain samples from the other populations within the canyon as well as the south side of Lake Mead before making taxonomic changes. The diversity of *A. californica* and *A. merriamii* should be explored further, but markers should be designed with the DNA from each species as the starting material, and then each could be tested in the other two species. Without question, the only efficient and cost-effective way to complete a study of this nature, particularly with endangered species that have little genetic work done or markers already developed, would be to start with 454-sequencing, rather than spending anytime with the other methods that were carried out at the beginning of this study.

*Arctomecon humilis* is a federally listed endangered species and is at risk from urbanization and development, off-road vehicles, rare habitat, and invasive species. Through the data obtained from this study it is evident that some sub-populations are in danger of becoming genetically isolated. Inbreeding depression can be caused by many factors, including ecological isolation, habitat fragmentation, decreases in populations size, reduced gene flow, random genetic drift, and the bottleneck effect (Hendrick and Kalinowski 2000, Keller and Waller 2002, Harper *et al.* 2000). A number of these factors seem to be at play around the Boomer Hill site in the west and Shinob Kibe in the northeast near Washington City. Each has a number of private alleles, but is also fixed for multiple loci suggesting that there is limited gene flow between these populations and the others (also see Figure 5-12).

While the genotype data shows that these populations are on a trend to become more isolated, admixture between certain regions is occurring. For most loci the populations in the center of St. George (Price Hills, Webb Hill, and Sun River), have the greatest amount of alleles present, and do seem to be providing a partial corridor between the easternmost and westernmost populations. Red Bluff is an important stepping-stone between Boomer Hill and the populations
around Price Hills. It should also be noted that the small cluster just southeast of Sun River (Red Wash) does seem to be genetically differentiated. This is likely due to the different soil type present at this location compared to those nearby. Monitoring of the primary pollinators and an assessment of the pollinator success should be incorporated.

Most of the current collection locations are already within the protected reserve system around St. George, UT, however any additional protection that can be provided in order to maintain as much genetic variability as possible is highly recommended. The city, state and federal government do seem focused on ensuring that this species is well protected, but the rare habitat (virtually only existing behind fences), makes a larger recovery effort difficult. Bear-poppies have likely always been rare, and they are highly adapted for success in their habitats. The main concern is that remaining populations and critical habitat continue to be protected.

Thus far it does seem as though the diversity of alleles has been captured within the reserve system, as the unprotected areas do not contain alleles that are not present in the protected areas (Figure 5-13).

Part of the success of the reserve system requires protecting all soil types that currently support *Arctomecon humilis*. This species is highly tied to its habitat, and based on a rough categorization of soil characteristics there is a wide variety of alleles in all soil types, except for the white gypsum soils or at least there is less variety found at those locations (Figure 5-14).

One last analysis that was proposed was to determine the level of alleleic diversity among different age-classes. However, due to the dates of collection and the lack of seedlings from year-to-year, the sample size of the seedling and juvenile categories were low. At the long-term demography site of Red Bluff, from 1987 to 2007 there were only three years with more than 100 seedlings, but thirteen years with fewer than ten new recruits (Harper and Van Buren 2004, and unpublished data). Thus the age-class difference is not robust due to the small sample size.
of seedlings. However, according to the genotype data that was observed, the seedlings did not differ significantly from the alleles found in the adult plants (Figure 5-15).

Ideally the level of protection for this species will continue, and the experimental data gathered by this study will be able to inform future conservation measures. This research is relevant, timely and valuable to managers of a recently established poppy reserve in Washington Co., Utah, owned by The Nature Conservancy and other responsible agencies.
Figure 5-1. Map of the *Arctomecon humilis* distribution around St. George, Utah.
Figure 5-2. Structure Harvester output of the Evanno method estimate of K.

\[ \Delta K = \text{mean}(\|L''(K)\|) / \text{sd}(L(K)) \]
Figure 5-3. Structure output of the genetic difference of the three *Arctomecon* spp., with differing levels of K.
Figure 5-4. Evanno plot for detecting the number of K groups that best fit the data (Evanno et al. 2005)
Figure 5- 5. The genetic clusters for *Arctomecon humilis* as detected by Structure and synthesized in Structure Harvester. **Note**: The cluster colors in each K are independent of each other.
Figure 5-6. PCA plot of individuals belonging to each region (K=4), with the westernmost populations of *Arctomecon humilis* exhibiting the greatest amount of differentiation based on genotype allele frequencies.
Figure 5-7. PCA based on Pairwise Shannon values (mutual information index) measuring differentiation among *Arctomecon humilis* populations based on genotype allele frequencies.
Figure 5- 8. a) Results from the AMOVA based on population. b) Results of the AMOVA incorporating regions. Analysis based on Arctomecon humilis genotype allele frequencies.
Figure 5-9. Frequency Distribution of Random PhiPT versus Observed PhiPT for 999 Permutations ($\Phi_{PT}=0.298, P=0.001$)
Figure 5-10. Mantel Results for Geographic Distance in km (GGD) vs Genetic Distance (GeneticD) based on pairwise similarities according to Arctomecon humilis genotype allele frequencies. $R^2$ in Mantel test cannot be evaluated as $R^2$ in the regular, bivariate sense (Mantel $R^2$ is always much lower).
Figure 5-11. PCA of the harmonic mean of Dest produced by SMOGD based on *Arctomecon humilis* genotype allele frequencies.
Figure 5-12. PCA of the K=10, genetic clusters based on *Arctomecon humilis* genotype allele frequencies. Points depict individual specimens with 95% CI ellipses for population clusters.
Figure 5-13. PCA of the *Arctomecon humillis* genotype allele frequencies categorized by whether the individual is found within a reserve or an unprotected area. Points depict individual specimens with 95% CI ellipses for population clusters.
Figure 5-14. PCA of the genotype allele frequencies categorized by the soils they were located on (1= White, 2= Brown, 3= Red, 6= Orange). Points depict individual specimens with 95% CI ellipses for population clusters.
Figure 5-15. PCA of the genotype allele frequencies categorized by age-class (1= seedlings, 2= juvenile, 3= adult). Points depict individual specimens with 95% CI ellipses for population clusters.
Table 5-1. Summary of Population sample size, Observed and Expected Heterozygosity, and F statistics by Population for Codominant Data based on genotype allele frequencies of *Arctomecon humilis*.

<table>
<thead>
<tr>
<th>Pop</th>
<th>N</th>
<th>Ho</th>
<th>He</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beehive_Dome</td>
<td>Mean</td>
<td>31.857</td>
<td>0.392</td>
<td>0.461</td>
</tr>
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<td></td>
<td>SE</td>
<td>0.097</td>
<td>0.068</td>
<td>0.065</td>
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<td>Boomer_Hill</td>
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<td>0.000</td>
<td>0.087</td>
<td>0.072</td>
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<td>0.520</td>
<td>0.586</td>
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<td></td>
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<td>Mean</td>
<td>30.000</td>
<td>0.471</td>
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<td>Shinob_Kibe</td>
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<td>0.297</td>
<td>0.413</td>
</tr>
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<td>40.786</td>
<td>0.473</td>
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<tr>
<td>Warner_Ridge</td>
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<td>0.392</td>
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<td>SE</td>
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<td>0.074</td>
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<td>Webb_Hill</td>
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<td>0.449</td>
<td>0.577</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.169</td>
<td>0.081</td>
<td>0.086</td>
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<tr>
<td><strong>Grand Mean and SE over Loci and Pops</strong></td>
<td><strong>Mean</strong></td>
<td><strong>33.629</strong></td>
<td><strong>0.434</strong></td>
<td><strong>0.531</strong></td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>SE</strong></td>
<td><strong>0.535</strong></td>
<td><strong>0.023</strong></td>
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</table>
Chapter 6 Conservation Biology

Conclusion

Research in the field of conservation genetics can reveal important insights for effective management of endangered or at-risk species. Genetic information or criteria based on such information is beneficial in deciding which populations deserve priority. This is of particular interest when dealing with small populations. In regards to evolutionary adaptation, small populations are more vulnerable because losses in genetic variation through genetic drift can adversely affect the level of genetic variation and thus the possibility of future adaptation. In addition, if inbreeding is occurring in isolated populations there is a good possibility that individual fitness will be reduced. The research that was presented herein helps to identify genetic threats such as inbreeding and limited genetic diversity, but it also aims to aid the delineation of a potentially new species, helps to designate management units across the range of Arctomecon humilis, and elucidates some of the metapopulation dynamics of this species. As a multi-locus study, measures of diversity, mean numbers of alleles per locus, the percentage of polymorphic loci, levels of heterozygosity, and allelic richness are all valuable. Additionally, by using hypervariable microsatellite markers the estimate of population divergence related to Nei’s parameters of $D_{st}$ and $G_{st}$ were able to find absolute and relative differentiation among the sampled populations.

Certainly the role of conservation genomics should not be purely an academic exercise, but should be used to inform the management of species. Due to the limited space and lack of suitable habitat, changes in Bear-poppy management will be restricted but should be adaptable. The Bureau of Land Management, the United States Fish and Wildlife Service, and The Nature Conservancy are already working together for the benefit of these species. In addition they have
already been working with local researchers and the public, which has made implementation of new changes and modifications of management practices more acceptable in the communities surrounding these species. Conservation management has to evaluate the needs of all stakeholders involved if it is to remain successful. Continuous monitoring of these species and their ecological interactions within their community is essential, primarily assessments of pollinator health, seed dispersal, herbivory in drought years, and levels of habitat sustainability and maintenance will help to ensure the future survival of these unique species.
Appendices

Appendix A. Research and Collection Permits

Desert National Wildlife Refuge
United States Department of the Interior
U.S. Fish and Wildlife Service
Special Use Application and Permit

1. Desert National Wildlife Refuge

2. Application Date: Jun 21, 2010

3. Period of Use Applying for:
From: Jun 23, 2010
To: Jul 4, 2010

4. Applicant Information:
Name: Joshua Simpson
Phone: 917-415-5814
Organization: City University New York
Fax:
Address: 424 West 110th St. #18D
email: jsimpson@nybg.org
City/State/Zip: New York City, NY 10025

5. Purpose of the Permit Application:
- [ ] Agriculture
- [x] Commercial Visitor Services
- [ ] Commercial Filming
- [ ] Other (describe)
- [x] Research/Monitoring
- [ ] Special Event

6. Describe the above activity as specifically as you can.
   - Include: Where the activity will take place (units, roads, trails), when (season, days, hours), how (methods, techniques, transportation), frequency (one time only, daily, occasionally), number of people/vehicles/boats, special needs/permissions.
   - Researchers may be required to supply a research proposal.

   I am conducting a genetic study on the genus Arctomecon (Passeridae). I will be obtaining one to two leaves (depending on their size), from approximately 30 individual plants across the population distribution. In the DNWR I would like to sample from Arctomecon meminii. This will occur up Alamo Road, near Deed Horse Road. I anticipate spending one full day (between June 23 and July 3) in the DNWR. I will be traveling alone by four-wheel drive, staying on the designated roads and then hiking from that point.
   - In order to get a random sample of the genetic diversity present in the population, I plan to sample from one end of the distribution to the other (by visual estimation). Sampled individuals will be at minimum 20 meters apart from each other.
   - Further details of the overall project can be found in the research proposal sent separately.

7. Print this form and return it to the refuge for processing. Do not fill out any information below this line.

For Official Use Only

Application approved: [x] yes [ ] no

Special Conditions
Personnel working under conditions of this permit must carry a copy of the permit while on the refuge.

Record of Payments:
[X] Payment exempt [ ] Partial payment [ ] Full payment

Amount of payment

Record of partial payments:

This permit is issued by the U.S. Fish and Wildlife Service and is subject to the terms, conditions, obligations, and restrictions, expressed or implied herein, and to the rules, conditions, and requirements contained in the review of the permit.

Permit Issued By: (signature and title)

Date: 6/21/10

Desert National Wildlife Refuge Manager

DMT Control No. 10154198 (expires 6/21/2011)
Grand Canyon Collection Permit

**Scientific Research and Collecting Permit**

Grants permission in accordance with the attached general and special conditions

United States Department of the Interior
National Park Service
Grand Canyon NP

**Study #:** GRCA-00059
**Permit #:** GRCA-2011-SCI-0015
**Start Date:** Apr 04, 2011
**Expiration Date:** Apr 04, 2012
**Coop Agreement #:** n/a
**Optional Park Code:** n/a

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**Name of Principal Investigator:**

*Name:* Wendy Hodgson  *Phone:* 480-481-8108  *Email:* whodgson@dbg.org

**Name of Institution Represented:**

Desert Botanical Garden

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**Co-Investigators:**

- **Name:** Andrew Salywon  *Phone:* 480-481-8107  *Email:* n/a
- **Name:** Dr. Robert Bye  *Phone:* n/a  *Email:* n/a
- **Name:** Josh Simpson  *Phone:* n/a  *Email:* jsimpson@nybg.org
- **Name:** Larry Hufford  *Phone:* n/a  *Email:* n/a

**Project Title:**

Study of the flora of Grand Canyon National Park and increasing the holdings of Grand Canyon National Park Herbarium

**Purpose of Study:**

The herbarium collection is needed for research and study, loan to scientists preparing monographic treatments, other taxonomic research, range extensions, and the assembly of biotic community lists and information on plant abundance, elevations, flowering and fruiting months, and invasions.

This study would continue to focus on poorly documented or represented taxa from primarily, although not exclusively, the Inner Gorge. Continued documentation of flowering and fruiting plants within the Canyon will augment considerably the Canyon's herbarium, at little or no financial cost to the Park. No listed endangered or threatened species will be collected, although it may be photographed with sufficient locality information included.

**Subject/Discipline:**

Vascular Plants

**Locations Authorized:**

Fieldwork and documentation will be throughout Grand Canyon region - inner gorge and north and south rims, with emphasis on areas with trails or routes and from Vulcan’s Throne upstream to Marble Canyon.

**Transportation Method to Research Site(s):**

Use of designated foot trails and open public roads is authorized. P.I. may join already scheduled river trips on a space-available basis.

**Collection of the Following Specimens or Materials, Quantities, and Any Limitations on Collecting:**

Mentzelia, Yucca, Cirsium, Heliotropium, Nolina, Agave, Aquilegia, all Cactaceae, Hesperoyucca, and Datura.

**Name of Repository for Specimens or Sample Materials if Applicable:**

Repository type: Permanently retained in National Park Service collection, maintained by NPS repository

Objects collected:

Quality herbarium specimens (no listed threatened or endangered) will be collected for Grand Canyon National Park herbarium, with duplicates (if appropriate) deposited in the Desert Botanical Garden Herbarium. Each specimen will have information such as locality (using a Global Positioning System), relative abundance, date of collection, collector's number, and species name. Photographs of cacti and agavaceae will also be made. Specimens will be sent to Grand

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Page 1 of 3
1. NAD 83 HARN is the UTM used by all researchers to ensure compatibility with all GRCA GIS layers.

2. PI agrees to utilize Leave No Trace techniques to minimize impacts to vegetation during collection and processing.

3. No vegetation disturbance will be made during the course of the fieldwork portion of this study.

4. Copies of all field notes, photographs, maps, reports and all data shall be archived at Grand Canyon National Park Museum Collection upon project completion and will be provided to the Research Coordinator within 6 months upon completion of this study.

These documents can be submitted electronically to grca_research_coordinator@nps.gov or via hard copy to:

Grand Canyon National Park Research Coordinator
1824 South Thompson Street, Suite 200, Flagstaff, AZ 86001

5. PIs will be required to submit datasets and reports from any previous permitted research before a new or renewal of a permit will be considered. The 90 day evaluation period for a research permit will not start until datasets and reports are received by the GRCA Research Coordinator.

6. Camping and Public Interaction Requirements

a. PIs and associated researchers will camp in areas that minimize public interaction and that are less desirable to the visiting public.

b. PIs and associated researchers will have professional contacts with the public and spend a reasonable amount of time explaining the purpose of their studies to the visiting public.

7. Safety Procedures

a. PIs and associated researchers will follow the safety procedures outlined in Appendix C of the 2008 Commercial Operating Requirements (p. 32-33) for helicopter evacuations of their trips and any other trip they may encounter that is in distress.

b. PIs will cease scientific data collection to ensure the safety and well-being of their trip or other trips they encounter in the park.

8. Consequences for Non-Compliance

Violating a term or condition of a permit issued pursuant to this section may also result in the suspension or revocation of the permit by the superintendent (56 CFR, Part 1, A§1.6(h) Permits)

9. PIs will meet with the GRCA staff as needed to discuss the conditions of their permit.

10. Follow your responsibilities and authorized activities of permit (as listed above), as well as Grand Canyon National Park (GRCA) regulations, and conditions for Research Permit holders as listed on GRCA research website.


12. Submit a Final Report when permit expires or within six months of completion of study. This report can be submitted electronically to grca_research_coordinator@nps.gov

or via hard copy to: Grand Canyon National Park Research Coordinator
1824 South Thompson Street, Suite 200, Flagstaff, AZ 86001

13. All Final reports and documentation resulting from this study are to be submitted to the park for review before distributing to the public/press.

14. GRCA staff is authorized to accompany research/sampling trips and provisions will be made for park participants.

15. If archeological materials and/or sites are encountered, all work must stop and the Research Office & Park Archeologist must be consulted and provided with exact locations and/or UTM coordinates of the sites/plots. Jane Rodgers can be contacted at (928) 606-5793 and Ronda Newton can be contacted at (928) 538-7447.

16. The Native American Graves Protection and Repatriation Act of 1990 (NAGPRA) assigns ownership and control of Native American Human remains, funerary objects, sacred objects, et al to the lineal descendants or affiliated Indian tribes. Consultation with affiliated tribes is required within a set time frame, with very specific processes that have been established through the NAGPRA review committee. Responsibility falls to the land manager and Principle Investigator regardless of the context of the discovery. All GRCA research projects and research personnel must be aware of the
17. In accordance with the Paleontological Resources Protection Act of 2009 (PL 111-11), resources collected from Federal lands are Federal property. All collected resources and copies of associated records will be preserved and made available for the public and researchers (Sec. 6304, C. 1-2). In addition, any resources and data and records collected will be stored and curated in an approved repository (Sec. 6305).

18. Researcher Liability Waiver

Accessing research areas and conducting research in Grand Canyon National Park entails various serious risks to human health or safety posed by the largely natural conditions of the park. These risks include, but are not limited to, possible exposure to steep, uneven terrain, unstable rocks or soils, extreme and unpredictable weather, flash floods or other weather-related events, unpredictable river or stream currents, dangerous wild animals and plants, difficult route-finding, and underground mine and cave-related risks. The Permittee is aware of these risks, and of other risks not listed above, and voluntarily and knowingly assumes all risks associated with accessing research areas and conducting research in the park.

In consideration of the National Park Service’s authorizing the Permittee to access research areas and to conduct research in Grand Canyon National Park, the Permittee hereby waives any and all claims, demands, and causes of action, and releases the United States and its employees and officers from any and all liability, arising out of or related to this authorization.

Furthermore, the Permittee hereby agrees to indemnify and hold harmless the United States and its employees and officers against any and all liability to third parties arising out of or related to the Permittee’s access to research areas or the conduct of research in Grand Canyon National Park. As part of this agreement, the Permittee agrees to reimburse the United States for its costs, including the costs of federal employees’ time, in investigating, defending, compromising, or otherwise resolving any claim, demand, or cause of action brought by any third party against the United States or its employees or officers arising out of or related to the Permittee’s access to research areas of the conduct of research in the park.

19. All collected specimens must be delivered to the park Museum Collections by March 31 each year.

20. Provide GRCA’s Research Permit Coordinator with the spreadsheet of field notes/location/specimen collection information.

I Agree To All Conditions And Restrictions Of this Permit As Specified
(Not valid unless signed and dated by the principal investigator)

I, ___________________________,
(Principal Investigator’s signature)

Date: ________________

THIS PERMIT AND ATTACHED CONDITIONS AND RESTRICTIONS MUST BE CARRIED AT ALL TIMES
WHILE CONDUCTING RESEARCH ACTIVITIES IN THE DESIGNATED PARK(S)
NY Botanical Garden Poppy Study Throughout Southern NV

Decision

It is my decision to approve the proposed plant study throughout Southern Nevada NV-052-TA-10-06, with the conditions of use listed in DOI-BLM-NV-010-010-010-CX which the applicant has agreed to follow.

Rationale:

The proposed action is in conformance with the Land Use Plan Nevada Las Vegas Resource Management Plan, October, 1998. Even though it is not specifically provided for, it is clearly consistent with the following LUPE decision(s) (objectives, terms, and conditions):

- Objective 88-4: Encourage the obtainment and dissemination of knowledge regarding the Mojave Desert ecosystem including desert tortoise biology.

This activity meets the following exception identified in the BLM list of actions categorically excluded from development of an Environmental Assessment or Environmental Impact Statement 518 D1 Chapter 2, Appendix 1 Department C.X. (1.5) non-destructive data collection, inventory (including field, aerial, and satellite survey and mapping), study, research and monitoring activities and does not meet any of the 12 exceptions to categorical exclusions.

Based upon review of available data and staff recommendations and analysis, I have determined the proposed action is a categorical exclusion and does not meet any of the exceptions in 518 D1V 2 Appendix 2. No further environmental analysis is required.

Authorizing Official:

[Signature]

Gayle Marz-Smith
APR Renewable Resources

Date: 5/18/10

Contact Person

For additional information concerning this Finding, contact Katie Kleinick, 702-513-5351
NY Botanical Garden Poppy Study Throughout Southern NV

Background

DOI-BLM-NV-S010-2010-0110-CX

BLM Office:

Nevada - Las Vegas Field Office
LLNVS00520

Lease/Serial/Case File No.: 

NV-052-UA-10-06

Proposed Action Title/Type:

Research Permit for NY Botanical Garden Poppy Study Throughout Southern NV

Location of Proposed Action:

This study will be conducted within the species populations throughout Southern Nevada. See attached maps along with list of legal descriptions. This permit authorizes activities on BLM lands only.

Description of Proposed Action:

Graduate student, Joshua Simpson from the New York Botanical Garden is requesting to collect data and leaf tissue samples from plant species *Arctomecon californica* and *A. merriamii* on public lands throughout Southern Nevada. This sampling will allow the comparison of the genetic variability in *A. californica* and *A. merriamii* to genetic variation of *Arctomecon humili* for which data collection is already underway.

Plants will be randomly selected for leaf tissue collection from across the entire range of the population being sampled. The aim is to collect leaf tissue (1-2 leaves) from a minimum of 30 individuals in each population with equal representation from multiple sources.

In addition to leaf tissue sampling, they will also be obtaining ecological data by setting up temporary transects that will be removed the same day following data collection. This will include community composition, density, cover, frequency and importance values.
The researcher plans to conduct these transects and do the leaf tissue sampling in June 2010. The length of time needed for sampling and setting up transects will be one to two consecutive days in each location (conducting three separate transects in each) from dawn until dusk.

Sampling and data collection will be carried out by 1 to 3 people utilizing a single four-wheel drive vehicle on existing authorized roads. Researchers will hike into study sites.

**Land Use Plan Conformance**

**Land Use Plan Name:**

Nevada - Las Vegas RMP

**Date Approved/Amended:**

October, 1998

**The proposed action is in conformance with the Land Use Plan, even though it is not specifically provided for, because it is clearly consistent with the following Land Use Plan decision(s) (objectives, terms, and conditions):**

- Objective SS-4. Encourage the obtainment and dissemination of knowledge regarding the Mojave Desert ecosystem including desert tortoise biology.

**Compliance with NEPA:**

The Proposed Action is categorically excluded from further documentation under the National Environmental Policy Act (NEPA) in accordance with 516 DM 2, Appendix 1,1.6:

- Nondestructive data collection, inventory (including field, aerial, and satellite surveying and mapping), study, research, and monitoring activities.

This categorical exclusion is appropriate in this situation because there are no extraordinary circumstances potentially having effects that may significantly affect the environment. The proposed action has been reviewed, and none of the extraordinary circumstances described in 516 DM 2 apply.

I considered the following conditions of use for the applicant to follow while carrying out their requested action:

1. All motorized vehicles are restricted to existing designated roads. Use is not permitted on any roads that access by the permittee will modify the condition of the road.
2. Speed limit of 25 mph will be maintained on all unposted dirt roads.
3. All litter, garbage and trash shall be removed from the site daily.
4. The painting of rocks or the establishment of permanent markers or improvements is prohibited.
5. Desert tortoises are protected by law. Handling or harassing is prohibited unless they are in imminent danger (e.g. from oncoming traffic).
6. Prior to moving a parked vehicle the tires and ground beneath the vehicle will be inspected for desert tortoises. If a tortoise is found, it must be allowed to move out of harms way on its own volition. If it has withdrawn it is considered in imminent danger and may be moved in accordance with approved handling techniques.
7. No permanent ground disturbance is authorized in association with this activity.
8. It is unlawful to disturb any archaeological sites or remove, relocate or bury archaeological artifacts. Care must be taken when visiting archaeological sites to avoid excessive walking on or crushing of archaeological deposits (middens) and surface manifestations.
9. Applicant must not disturb archaeological and historical sites, including, but not limited to, petroglyphs, ruins, historic buildings, and artifacts. Any cultural artifacts or vertebrate paleontological materials (including fossil trackways) discovered through permitted operations must be left in place and the BLM notified. The granting of this special use-authorization does not give the permit holder permission to collect cultural or paleontological resources.
10. Participants are required to inspect, remove, and bag for disposal, all seed and vegetation from personal clothing and equipment before entering all sites and upon leaving all sites. These stipulations are intended to reduce the transportation and establishment of noxious weeds to new locations.
11. If the field and collection area is near a mining operation, the researcher needs to contact the mine operator and gain permission to cross claims or property as some operations do blast from time to time and many have large heavy equipment that could easily injure or kill someone.
12. If access to the study areas includes crossing over private lands with no public road (ingress/egress) permission should be obtained from the private landowner.
13. The proposed action is located within and adjacent to Muddy Mountains Wilderness, Lime Canyon Wilderness and Sunrise Mountain Instant (Wilderness) Study Area. Researchers will utilize Leave No Trace practices while within these areas.
14. A portion of one study area is within the Gold Butte Herd Management Area (HMA). If wild burros are encountered they should not be harassed (fed, pet, ridden, or chased).
15. A summary of the findings, when available, will be submitted to the BLM.

**Consultation and Coordination**

The following BLM specialists reviewed, provided comments and/or assisted in the preparation of this document:

- Lisa Christianson, Air Quality Specialist
- Susanne Rowe, Archeologist
- John Evans, Planning and Environmental Coordinator
- Brenda Warner, Lands Specialist
- George Varhalmi, Minerals
- Nora Caplette, Noxious Weeds
- Sarah Peterson, Hydrologist
- Fred Edwards, Vegetation
- Lauren Brown, Visual Resources
- Sendi Kalcie, Wilderness
• Krystal Johnson, Wild Horse and Burro Specialist
• Mark Slaughter, Wildlife Biologist
• Chris Linehan, Recreation Specialist
• Greg Marfil, Fire Specialist

Applicant Signature

KEEP THIS AUTHORIZATION WITH YOU AND SHOW IT TO ANY BLM RANGER ON REQUEST

I have read and agree to the conditions listed above.

Joshua Simpson, New York Botanical Garden
718-817-6062
jsimpson@nybg.org

Approval and Contact Information

Authorizing Official:

[Signature]

5/17/10

BLM Research Coordinator

Gayle Marr-Smith
AFM Renewable Resources

Contact Person

For additional information concerning this CX review, contact Katie Kleinick at (702) 515-5351.
Figure 11. The known global extent of Arctomecon merriamii, white beargrass.
<table>
<thead>
<tr>
<th>Species</th>
<th>NAME</th>
<th>MAP_QUAD</th>
<th>LATITUDE</th>
<th>LONGITUDE</th>
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<td>Arca</td>
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<tr>
<td>Arca</td>
<td>PASCO Mine E; Government Wash</td>
<td>T20S R64E 58/9/18/17</td>
<td>36.2</td>
<td>-114.5</td>
</tr>
<tr>
<td>Arca</td>
<td>Gale Hills; Calvillo Bay</td>
<td>T20S R65E 58/9</td>
<td>36.2</td>
<td>-114.5</td>
</tr>
<tr>
<td>Arca</td>
<td>Bitter Spring NW; Bitter Spring</td>
<td>T19S R67E 58/17</td>
<td>36.2</td>
<td>-114.5</td>
</tr>
<tr>
<td>Arca</td>
<td>Echo Hills NW; Bitter Spring</td>
<td>T19S R67E 517/20/29-30</td>
<td>36.2</td>
<td>-114.5</td>
</tr>
<tr>
<td>Arca</td>
<td>Bitter Ridge; Devils Throat</td>
<td>T17S R69/70E 51,11-13/7,18</td>
<td>36.4</td>
<td>-114.5</td>
</tr>
<tr>
<td>Arca</td>
<td>Lime Ridge E; Devils Throat</td>
<td>T17/18S R69E 538/1,12</td>
<td>36.3</td>
<td>-114.5</td>
</tr>
<tr>
<td>Area</td>
<td>Location Details</td>
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<td>Latitude</td>
<td></td>
</tr>
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<td>----------------------------------------------------------------------------------</td>
<td>-----------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>Arca</td>
<td>Government Wash mouth; Government Wash</td>
<td></td>
<td>36.13190</td>
<td></td>
</tr>
<tr>
<td>Arme</td>
<td>Ash Meadows</td>
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<td></td>
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</tr>
<tr>
<td>Arme</td>
<td>Devils Hole</td>
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<td>Arme</td>
<td>Last Chance Range</td>
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<tr>
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<td>Stewart Valley</td>
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<tr>
<td>Arme</td>
<td>Pahrump Valley</td>
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<tr>
<td>Arme</td>
<td>South Delamar Mountains</td>
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<tr>
<td>Arme</td>
<td>East of Spring Mountains</td>
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<td></td>
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<td>Las Vegas Valley</td>
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<td>Calico Hills</td>
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<td>Bird Spring Range</td>
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<td>Speculator Range</td>
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<td>Desert National Wildlife Refuge</td>
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<tr>
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<td>Indian Springs</td>
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<tr>
<td>Arme</td>
<td>Pint Water Range</td>
<td>-115</td>
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<tr>
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<td>Black Hills</td>
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</tr>
<tr>
<td>Arme</td>
<td>Three Lakes Valley</td>
<td>-115</td>
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</table>
The original Bureau of Land Management and U.S. Fish and Wildlife Service permit that I was amended onto in order to collect *Arcotmecon humilis* in Utah.
Shrubby reed-mustard *Schoenocrambe suffrutescens*
Siler pincushion cactus *Pediocactus sleri*
Uinta Basin hookless cactus *Sclerocactus gaucus*
Ute ladies'-tresses *Spiranthes diluvialis*
Welsh's milkweed *Asclepias welshii*
Winkler cactus *Pediocactus winkleri*
Wright fishhook cactus *Sclerocactus wrightiae*
SPECIAL TERMS AND CONDITIONS FOR  
Bureau of Land Management  
Utah State Office

Species: Barneby ridge-cress *(Lepidium barnebyi)*  
Barney reed-mustard *(Schoenocrambe barnebyi)*  
Clay reed-mustard *(Schoenocrambe argillacea)*  
Dwarf bear poppy *(Arctomecon humilis)*  
Holmgren milk-vetch *(Astragalus holmgreniorum)*  
Jones cyclademia *(Cyclademia humilis jonesii)*  
Kodaehrome bladderpod *(Lesquerella tumulosa)*  
Last Chance townsendia *(Townsendia aprica)*  
Maguire daisy *(Eryngium maguirei)*  
Navajo sedge *(Carex spectucola)*  
San Rafael cactus *(Pediocactus despainii)*  
Shivwits milk-vetch *(Astragalus ampullarioides)*  
Shrubby reed-mustard *(Schoenocrambe suffrutescens)*  
Siler cushion cactus *(Pediocactus sileri)*  
Utah ladies'-tresses *(Spiranthus diurialis)*  
Welsh’s milkweed *(Asclepias welshii)*  
Winkler cactus *(Pediocactus winkleri)*  
Wright fishhook cactus *(Sclerocactus wrightiae)*  
Uinta Basin hookless cactus *(Sclerocactus glaucus)*

This permit authorizes the following activities in Utah, through December 31, 2012, to enhance recovery, survival, propagation, and scientific research under the following conditions:

E. **Permittee named on the face of this permit is responsible for ensuring that the activities of all individuals are in compliance with the terms and conditions of this permit. Only individuals on the attached List of Authorized Individuals are approved to conduct activities pursuant to this permit.**

F. Extreme care shall be taken to avoid direct trampling or impacts to the above listed plant species, all species listed under the Endangered Species Act, and plants and animals found on the BLM’s sensitive species list for Utah and excessive trampling or use within all listed and sensitive species habitat is to be avoided.

G. Collect voucher specimens:

1. All land locations for collected vouchers shall be discussed with the Project Leader (see attached list) prior to collection and will be identified by 7½" Quad name and Township and Range in publicly available documents, such as herbarium labels, but not to the specifications that may endanger the
species to adverse activities, such as illegal collection. No Universal
Transverse Mercator (UTM) coordinate system is to be used in public
documents.

2. To the degree possible, voucher specimens or individuals that are collected
shall be done by methods in which no roots are to be removed and no more
than 2 flowering stems shall be taken or collected from an individual plant.

An exception for authorization for collection that would exceed 1% of the
population, must be obtained in advance of collection by expressed written
permission of the Project Leader (see attached list) and may be permitted
under certain conditions.

3. Specimens shall be handled in accordance with standard museum practices.
Before the deadline for annual reports under the requirements of this
permit, all preserved specimens shall be properly labeled and preserved,
and based on mutual agreement with the Project Leader (see attached list)
either deposited with a designated herbarium or incorporated into an
educational program. The permittee shall supply a copy of this permit to
validate that the specimens were taken pursuant to a permit.

4. A collection for up to 1 plant individual per each above listed species at a
newly discovered site within the State of Utah as voucher specimens, not to
exceed 1% of the population at new sites, not to exceed 1 new site per
above listed species, per year, not to exceed 4 plants per species during the
life of the permit.

An exception for authorization for collection that would exceed 1% of
the population, must be obtained in advance of collection by expressed
written permission of the Project Leader (see attached list) and may be
permitted under the circumstances where newly discovered population
may exhibit important or unique taxonomic characteristics, necessary
for additional examination by botanical experts and herbarium
representation (maximum permitted collection is 1 plant of unique
character per population per year, not to exceed 2 unique plant
representatives per species during the life of the permit).

5. A collection for up to 1 plant individual per each above listed species
within the State of Utah, found at any known site on BLM lands, not to
exceed 1% of the population at a given site for educational purposes, use, or
display by BLM, (maximum permitted 1 collection per each above listed
species solely for educational purposes, use, and/or display).

H. Collect seeds for storage and research. Seed collection directly from plants shall
not exceed 6,000 seeds per population, per year, per individual species with the
following restrictions:
1. Center for Plant Conservation (CPC) seed collecting guidelines will be followed. Seeds collected and not used for research are to be sent to an approved CPC institution for storage. To determine the appropriate CPC institution, please contact the Project Leader (see attached list).

2. Seed shall be collected only from the BLM lands.

3. No more than 10% of the plants within a population will have seed collected and no more than 100 plants within a population are to have seed collected within a given year.

4. Seed collections will not exceed 50% of the annual seed production of an individual plant and will not be done where fewer than 20 plants are present at a site without the expressed written permission of the Project Leader (see attached list).

5. Collection activities are limited to occur no more than 2 years, per above listed species in a 5-year period.

I. Conduct seed research. The following research activities are permitted: Seed catchments to determine seed bank longevity, soil samples which include seeds, seed testing for germination rates, fruit manipulation to determine seed counts and condition of seeds, and marking or manipulating of seed studies to determine predation. The following restrictions apply:

1. The number of seeds collected from surface soil and/or in the process of soil sampling will be documented and shall not exceed 500 seeds per above listed species. Should the number of seeds collected within soil samples exceed 500 seeds, the permittee will contact the Project Leader (see attached list). To the degree an additional amount of seed is collected from within the soil, a reduction of seeds taken directly from plants may be determined necessary.

2. In cases where possible, all generated seedlings not destroyed in research will be grown until their size/vigor are sufficient for transplanting them either in the subpopulation from which the seeds were originally collected or at a recognized CPC institute for public education and display. To determine the appropriate CPC institution, please contact the Project Leader (see attached list).

3. Experimental research is limited to the above seed collection stipulations for seeds collected directly from the wild. Additional seeds gained through experimental propagation are not limited in use, but recommended that a minimum of 5% of greenhouse or non-wild produced seed is sent to an approved CPC institution for storage.
J. Conduct plant identification and tagging:

1. To the degree possible, plant identification and tagging shall be done by methods in which no harm is done to individual plants.

2. Harm to individual plants is limited to tissue damage that shall not result in mortality or decrease longevity of an individual plant.

3. In the event that a plant is accidentally damaged or destroyed, the permittee shall include: A report of the circumstances that led to the damage or destruction. Describe the changes in activity protocols that will be implemented to reduce the likelihood of such future damage or destruction from happening again. If sufficient material is available, preserve any dead plant materials in accordance with standard museum practices. Before expiration of the permit, all preserved specimens shall be properly labeled and deposited with a designated depository. The permittee shall supply a copy of this permit to validate that the specimens were taken pursuant to a permit.

K. Conduct DNA/genetic work. DNA/genetics work may include leaf, petal, and/or tissue removal or damage. The following restrictions apply:

1. Removal of leaf, petal, or floral material for DNA/genetic work is to be limited to collection methods in which no harm long term harm is done to individual plants and should represent the least amount of harm necessary for research.

2. Harm to individual plants is limited to above ground plant parts and shall not result in the mortality or decrease in longevity of an individual plant.

3. In the event that a plant is accidentally damaged or destroyed, the permittee shall include a report of the circumstances that led to the damage or destruction. The report shall describe the changes in activity protocols that will be implemented to reduce the likelihood of such future damage or destruction from happening again. If sufficient material is available, dead plant materials shall be preserved in accordance with standard museum practices. Before expiration of the permit, all preserved specimens shall be properly labeled and deposited with a designated depository. The permittee shall supply a copy of this permit to validate that the specimens were taken pursuant to a permit.
I. Transportation of plant materials:

1. Transportation is limited to within the State of Utah for propagation or research. Transportation of materials to recognized research facilities outside of Utah is based on written permission through the Project Leader (see attached list).

2. The permittee is responsible to provide the Service written documentation of all activities and resulting research within and outside of Utah and ensuring these activities fall within this permit. Failure of this will result in the revocation of the permit.

3. No transportation shall be made for commercial purposes.

5. Collection of no more than 6 individuals of above listed plants containing roots are to be removed from wild populations on BLM lands for propagation or research (maximum permitted take 6 plants per above listed species in a 5-year period).

6. Transportation of seeds, plant tissue, and above ground parts of individuals will follow all applicable guidelines within the permit.

7. All land locations for transported plant materials will be discussed with the Project Leader (see attached list) prior to collection and will be identified by 7 1/2" Quad name and Township and Range in annual permit and publicly available documents, such as herbarium labels, but not to the specifications that may endanger the species to adverse activities, such as illegal collection. No Universal Transverse Mercator (UTM) coordinate system is to be used in public documents.

8. Specimens are to be handled in accordance with standard museum practices. Before the deadline for annual reports under the requirements of this permit, all preserved specimens shall be properly labeled and preserved and based on mutual agreement with the Project Leader (see attached list) either deposited with a designated herbarium or incorporated into an educational program. The permittee shall supply a copy of this permit to validate that the specimens were taken pursuant to a permit.

9. In the event that a plant is accidentally damaged or destroyed, the permittee shall include: A report of the circumstances that led to the damage or destruction. Describe the changes in activity protocols that will be implemented to reduce the likelihood of such future damage or destruction from happening again. If sufficient material is available, preserve any dead plant materials in accordance with standard museum practices. Before expiration of the permit, all preserved specimens shall be properly labeled.
and deposited with a designated depository. The permittee shall supply a copy of this permit to validate that the specimens were taken pursuant to a permit.

M. Conduct floral manipulation for pollination work:

1. Floral manipulation of individual plants is limited to above ground plant parts and shall not result in the mortality or decrease in longevity of an individual plant.

2. Removal of leaf, petal, stem, or floral material is to be limited to methods in which no long term harm is done to individual plants and should represent the least amount of harm necessary for research.

3. Manipulations of individual plants for pollination work shall not exceed 200 individuals range-wide, shall to the degree possible represent 1 in every 2 adjacent plants, and not exceed 50% of any site population.

4. Manipulation may include emasculation. No more than 1 in 5 or 20% of the total individuals in any 1 study site may be emasculated, not to exceed 50 plants range-wide, unless approved in written request by the Project Leader (see attached list).

N. Investigation of seed and fruit production:

1. All associated requirements within this permit for seeds and plant individuals must be applied.

2. Fruit manipulation of individual plants is limited to above ground plant parts and shall not result in the mortality or decrease in longevity of an individual plant.

3. Removal of leaf, petal, stem, or floral material is to be limited to methods in which no harm long term harm is done to individual plants and should represent the least amount of harm necessary for research.

4. Manipulations of seed and/or fruit of individual plants shall not exceed 200 individuals range-wide, shall to the degree possible represent 1 in every 2 adjacent plants, and not exceed 50% of any site population.

O. The allotted amount of mortalities authorized by this permit is 13 plants per each individual species over the course this permit. A reduction of take equal to each mortality that may occur as incidental or accidental to the below activities is required. In the event that more than the allotted mortalities occur, permitted activities must immediately cease. The Project Leader and the Resident Agent in Charge (see attached list) must be contacted within 24 hours. The Project Leader
must give approval before permitted activities may begin again. Disposition of mortality shall be at the direction of the Project Leader. Under this permit the collection or removal of roots is considered to be equivalent to the collection of an individual under all restrictions listed within this permit. Any exception to this or other below stipulations must be obtained in advance of collection or research activity by expressed written permission by Project Leader.

Coverage under this permit is provisional under the following restrictions:

P. You shall obtain the required permits and conduct your activities in compliance with all applicable laws and regulations of the State of Utah, and those Federal or tribal agencies upon whose lands you work. This permit does not grant the right of trespass. Such permission must be obtained from private landowners or the land management agency.

Q. Plant species referenced herein or their progeny shall not be sold, donated, or transferred without written authorization from the Project Leader (see below). Species and/or parts of species that are taken remain the property of the U.S. Fish and Wildlife Service unless otherwise indicated.

R. Annual reports:

1. An annual report of activities shall be submitted to the Project Leader and Permit Coordinator (see attached list) by December 31 annually. Failure to comply with reporting requirements may result in non-renewal or suspension/revocation of this permit.

2. The annual report shall include, but not be limited to:

   i. Summary presentations and brief discussions of significant research results;

   ii. Maps and/or descriptions of locations sampled will be discussed with Project Leader (see attached list) prior to release of information to public and/or be identified by 7½" Quad name and Township and Range in publicly available documents, such as herbarium labels, but not to the specifications that may endanger the species to adverse activities, such as illegal collection. No Universal Transverse Mercator (UTM) coordinate system is to be used in public documents;

   iii. The results of all sampling efforts, including estimates of population sizes;
iv. Numbers of individuals incidentally killed, including dates, locations, circumstances, and depository receiving the preserved specimen(s);

v. Other pertinent observations made during sampling efforts regarding the status or ecology of the species;

vi. Planned future activities if authorized under this permit.

S. If you wish to continue work with the above species after the expiration of this permit, your request for permit renewal must be received by the Permit Coordinator (see attached list), on or before December 31, 2011. Meeting this requirement allows you to continue authorized activities until your renewal application is acted upon. If this requirement is not met, this permit becomes invalid on the date of expiration. Any new activities or changes in activities with threatened or endangered species will require your permit to be amended. You are not authorized to conduct any new activities or to change any permitted activities until you have requested and have received a new or an amended permit.

T. Please reference permit number TE-165829 when submitting annual reports or other correspondence regarding this permit.

List of Contacts:

Project Leader, Ecological Services, 2369 West Orton Circle, West Valley City, Utah 84119, telephone 801-975-3330

Resident Agent in Charge, Law Enforcement, 9297 South Wadsworth Blvd., Littleton, Colorado 80128, telephone 720-981-2777

Permit Coordinator, Ecological Services, P.O. Box 25486, Denver Federal Center, Denver, Colorado 80225, telephone 303-236-4256
LIST OF AUTHORIZED INDIVIDUALS FOR
Bureau of Land Management
Utah State Office

Individuals authorized to conduct activities pursuant to this permit:

Ron Bolander, Holly Beck, Daryl Trotter, Jesse Salix, Lisa Church, Maria Ulloa-Cruz, Amber Huges, Karl Ivory, Bob Douglas, Debbie Clark, Renne Van Buren, Bernard Tait, Duane Atwood, Bruce Glison, Megan Robinson, Katie Moon, Dorde W. Woodruff, Tom Mathies, and Leigh Johnson.

Supervised individuals may conduct activities pursuant to this permit only under the direct, on-site supervision of an appropriate above-named independently authorized individual.

Each named individual shall be responsible for compliance with the terms and conditions of this permit. Permittee(s) named on the face of this permit is responsible to ensure that the activities of all individuals listed herein and their subordinates are in compliance with the terms and conditions of this permit.

Date 2/18/09

Deputy ARD – Fisheries-Ecological Services

This List of Authorized Individuals (List) is valid only if it is dated on or after the permit issuance date. This permit will be considered invalid without this List.

To request changes to this List, the permittee shall submit a written request to the Project Leader (see attached list). The request shall include the name of each individual to be appended to the List; a resume of qualifications of each person to be appended to the List detailing their experience with each species and type of activity for which authorization is requested; the names and phone numbers of a minimum of two references; and the names of individuals to be deleted from the List, if applicable.
Appendix B. This series of maps with the allele frequencies shown as pie charts along with the *Arctomecon humilis* branch of the Majority Rule consensus tree resulting from all the gene regions. Note: Pie charts are in their relative position as depicted in Figure 5.1, and in the same order as the figures in Appendix C.
Appendix C. Series of allele frequencies for each locus (listed in the same order as Appendix B) for all of the *Arctomecon humilis* genetic clusters. In relation to the locations depicted in Figure 5.1, the genetic clusters across the horizontal axis are as follows; 1) Beehive Dome 2) Sun River and Bloomington 3) Boomer Hill, South Butte, and Gnarly 4) Price Hills 5) Red Bluff 6) Red Wash 7) Shinob Kibe 8) Warner Ridge 9) Webb Hill 10) White Dome
Appendix D. Perl and R Script used to analyze data and generate figures.

PopGenReport.R: used to create tables and graphs of genotype allele frequencies.

```r
library(PopGenReport)
library(adeegenet)
setwd("~/Documents/Rscript/PopGenReport")

###PopGen: A simple way to analyse and visualize population genetic data
setwd("~/Documents/Rscript/PopGenReport") #set working directory
read.csv("PopGenArhuOnly418v3.csv", sep="","") -> ArhuAlleles #my data file
ArhuAlleles[1:10,] #columns with alleles
newsetArhuAllelesB <- read.genetable((paste("PopGenArhuOnly418v3.csv", sep="","")), ind=1, pop=2, lat=3, long=4, other.min=5, other.max=7, oneColPerAll=TRUE, sep="/", ncode=6)
newsetArhuAllelesB #make sure data read in correctly
summary(newsetArhuAllelesB)
popgenreport(newsetArhuAllelesB, mk.complete=TRUE, path.pgr = getwd(), fname = "popgenArhuB")

###individual analyses that can be done
#popgenreport(newsetArhuAllelesB, mk.counts=TRUE, mk.map=TRUE, mk.allele.dist=TRUE, path.pgr = getwd(), fname = "popgenArhuCount")
#popgenreport(newsetArhuAllelesB, mk.map=TRUE, path.pgr = getwd(), fname = "popgenArhuMap")
#popgenreport(newsetArhuAllelesB, mk.allele.dist=TRUE, path.pgr = getwd(), fname = "popgenArhuAllele")
popgenreport(newsetArhuAllelesB, mk.hwe=TRUE, path.pgr = getwd(), fname = "popgenArhuHW")
popgenreport(newsetArhuAllelesB, mk.locihz=TRUE, path.pgr = getwd(), fname = "popgenArhuLocihz")
popgenreport(newsetArhuAllelesB, mk.fst=TRUE, path.pgr = getwd(), fname = "popgenArhuFst")
popgenreport(newsetArhuAllelesB, mk.gd.smouse=TRUE, path.pgr = getwd(), fname = "popgenArhuSmouse")
popgenreport(newsetArhuAllelesB, mk.gd.kosman=TRUE, path.pgr = getwd(), fname = "popgenArhuKos")
popgenreport(newsetArhuAllelesB, mk.differ.stats=TRUE, path.pgr = getwd(), fname = "popgenArhuDiffer")
```

PCQ program in R; Dr. Dwight Kincaid releases the copyright on the following R code.

```r
# Name of this file:  PCQ 2.R A program in R (www.r-project.org)
# =============================================================================
# # Author: Professor Dwight Kincaid
# # IMPORTANCE VALUES in quantitative ecological plant inventory
# # version 19 March 2011 Lehman College, City University of New York
# # Bronx, New York 10468 USA 718.960.8235/.8651
# # REQUIRES: base R only
# # NOTE: Reads a csv file: col names and order of cols MUST BE- PCQ, sp.ID, DBH
# # NOTE: Enter raw data in the 3 vectors below. Comment-out the 3 below and add your own.
# # NOTE: To preserve output text in R Console, copy/paste it out.
setwd("~/Documents/Rscript/Kincaid")

#I am changing all of the DBH to Diameter, and then will save this as a separate file from Kincaid's original 'PCQ_2.R'
my.frame <- read.csv("ArhuPCQdataForKincaidR.csv"); attach(my.frame);
summary(my.frame)

s <- length(unique(sp.ID)) # N of species - a scalar
n <- length(diameter) # N of trees or plants - a scalar
pts <- length(unique(PCQ)) # N of PCQ points or quadrats - a scalar
basal <- pi * (diameter/2)^2 # diameter converted to basal area - a vector

my.frame <- data.frame(PCQ,sp.ID,diameter,basal) # add basal area column to obs data

# -------------------------------
# RELATIVE DENSITY in %
den <- tapply(diameter,sp.ID,length) # density
rel.d <- (den/n)*100 # rel.density

# -------------------------------
# RELATIVE FREQUENCY in %, the vector 'freq' is absolute freq
my.fun1 <- function(PCQ) length(unique(PCQ))
```
freq <- tapply(PCQ,sp.ID,my.fun1); rel.freq <- (freq/sum(freq))*100

# RELATIVE DOMINANCE in % of total basal area
basal.by.species <- tapply(basal,sp.ID,sum)
rel.dom <- (basal.by.species/sum(basal))*100

mean.diameter <- tapply(diameter,sp.ID,mean)  # get vector of mean diameter per taxon

# IMPORTANCE VALUE as rel.den. + rel.freq. + rel.dom.
IV <- rel.d + rel.freq + rel.dom
aa <- order(IV,decreasing=TRUE)

# sort vectors by order of IV value
rel.d <- rel.d[aa]; rel.freq <- rel.freq[aa]; rel.dom <- rel.dom[aa]
den <- den[aa]; freq <- freq[aa]; mean.DBH <- mean.diameter[aa]
IV <- rel.d + rel.freq + rel.dom  # get IV again, using sorted vectors

# CUMULATIVE values
C.IV <- cumsum(IV); C.d <- cumsum(rel.d); C.freq <- cumsum(rel.freq); C.dom <- cumsum(rel.dom);

# construct the data frame of results
spacer <- "---"
IV.frame <-
data.frame(den,freq,mean.diameter,spacer,rel.d,C.d,rel.freq,C.freq,rel.dom,C.dom,IV,C.IV)

# print results to R Console
options(digits=4)
lf <- function() print("",quote=FALSE);lf();lf()
d.line <- function(){
  print("======================================================================
  ====",quote=F)}
d.line();lf();print(" Output from 'PCQ 2.R' by Professor Dwight Kincaid,
dkincaid49@yahoo.com",quote=F)
lf();d.line();lf(); note1 <- paste("This run: ",date());
print(note1,quote=F); lf()
print(paste("NUMBER of SPECIES:",s),quote=F);lf()
print(paste("NUMBER of SHRubs:",n),quote=F);lf()
print(paste("NUMBER of SAMPLING UNITS:",pts),quote=F);lf()

print("The raw data is listed. PCQ stands for sampling unit: quadrat, PCQ quadrant, etc.");quote=F);lf()
print(my.frame);lf()

print("Absolute density, absolute frequency per sampling unit, mean diameter followed by --",quote=F);
print("rel. density, rel. frequency, rel. dominance(basal), Importance Value",quote=F)
print("and cumulative values for rel.den, rel.freq, rel.dom and IV.");quote=F);lf()
print(IV.frame);lf()
print("The first column above is species ID integer.");lf()

# ------------------------------------
# construct graphs

x.str <- "Species by IV Rank"; cl <- "wheat"; b.label <- rownames(IV.frame)
g1 <- "ECOLOGICAL DOMINANCE"; g2 <- "Abundance"; g3 <- "Distribution"; g4 <- "Size"

par(mfrow=c(2,2))
barplot(IV,col=cl,ylab="Importance Value (0-300)",ylim=c(0,max(IV)+.15*max(IV)),xlab=x.str,main=g1,names.arg=b.label)
barplot(rel.d,col=cl,ylab="Relative Density",ylim=c(0,max(rel.d)+.15*max(rel.d)),xlab=x.str,main=g2,names.arg=b.label)
barplot(rel.freq,col=cl,ylab="Relative Frequency",ylim=c(0,max(rel.freq)+.15*max(rel.freq)),xlab=x.str,main=g3,names.arg=b.label)
barplot(rel.dom,col=cl,ylab="Relative Dominance (Basal Area)",ylim=c(0,max(rel.dom)+.15*max(rel.dom)),xlab=x.str,main=g4,names.arg=b.label)
par(mfrow=c(1,1))

print("end   PCQ 2.R",quote=F);d.line()
Diversity Indices Program in R

# diversity 2.R    ver. 23 Nov. 2011   by   Dwight Kincaid
#
# USER enters frequencies with the lowest frequency as 1. Zeros not
# permitted.
# Comment-in, comment-out one data set for the run.
# File may be submitted as Source Code at R Console or copy/pasted as blocks.
# Don't forget to initialize NS as desired.
# Only base R is used.

rm(list=ls())           # delete all objects in RAM at R Console, comment-out
                        # as desired

NS <- 100000           # number of bootstrap samples; change as desired, begin low

# -----------------------------------------------------------------------------
# data of Hough(1936) on abundance of large trees in a PA forest,
# as analyzed on p. 364 of Krebs(1989) Ecological Methology 
# -----------------------------------------------------------------------------
# abundances for hemlock, beech, yellow birch, sugar maple, black birch, red
# maple, 
# black cherry, white ash, basswood, yellow poplar, magnolia

Hough <- c(1940,1207,171,134,97,93,34,22,15,7,4)

# -----------------------------------------------------------------------------
# Arctomecon humilis PCQ freq data of Joshua Simpson
# -----------------------------------------------------------------------------
#PCQ DATA, select one set to run at a time
ArhuAllPCQ_StGeorge <-
c(1,54,62,8,3,11,6,2,1,3,62,37,11,33,2,1,31,3,22,1,1,1,1,9,2,4,5,1,1,8)
ArhuPCQ_WhiteDome  <- c(11,8,2,3,12,11,11,6,1,8,1)
ArhuPCQ_SunRiver   <- c(5,1,1,1,1,6,3,2,2,1,3,1)
ArhuPCQ_PriceHills <- c(1,2,5,1,3,1,5,1,2,1,1,3)
ArhuPCQ_RedWash    <- c(7,3,2,1,6,3,5,3,1,1)
ArhuPCQ_BoomerHill <- c(1,5,5,3,9,1,1,1,1,1)
ArhuPCQ_WarnerRidge <- c(8,13,1,2,14,1,5,7,2,2,1)
ArhuPCQ_BeehiveDome<- c(7,13,1,2,3,14,1,2,12,2,2,2,1,1,1,1,1)
ArhuPCQ_ShinobKibe <- c(4,1,1,2,3,3,6,2,1,6,1)
ArhuPCQ_RedBluff   <- c(9,18,1,10,4,5,1,1,1,3)

# from Nested Frequency plots
ArhuAllNested_StGeorge <-
c(21,1,18,13,3,3,1,4,1,1,10,27,4,8,2,4,34,4,32,2,5,7,25,17,1,1,2,1,6,5,8,2,1,
   3,2)
ArhuNest_WhiteDome    <- c(5,1,3,3,4,6,4,5,2,2,5,1,3,4,1)
ArhuNest_SunRiver     <- c(1,1,2,1,3,6,2,1,2,1,1)
ArhuNest_PriceHills  <- c(3,1,1,4,6,1,3,9,10,1,1,5,1,2,1,1)
ArhuNest_RedWash    <- c(5,1,2,3,1,1,2,6,5,5,4,2,3,1)
ArhuNest_BoomerHill <- c(2,1,1,1,5,2,8,1,1,3)
ArhuNest_WarnerRidge<- c(3,1,1,4,5,3,1,2,3,2,1)
ArhuNest_BeehiveDome<- c(4,8,4,1,1,1,2,2,1,1,10,3,2,1,1)
ArhuNest_ShinobKibe <- c(3,2,4,1,2)
ArhuNest_RedBluff   <- c(4,2,3,3,1,2,3,7,1,2,1)

# --------------------------
# some simple, testing data
# --------------------------
tester1  <- c(5,5,5,5)
tester2  <- c(100,1,1,1,1,1)
tester3  <- c(5,5,5,5)
stilling.p.434 <- c(50,30,10,9,1)
stilling.p.433 <- c(100,50,30,20,1)

# ---------------------------------------------
# decide which data set to run while commenting-out all others
# ---------------------------------------------

#y <- Hough
#y <- ArhuAllPCQ_StGeorge
#y <- ArhuPCQ_WhiteDome
#y <- ArhuPCQ_SunRiver
#y <- ArhuPCQ_PriceHills
#y <- ArhuPCQ_RedWash
#y <- ArhuPCQ_BoomerHill
#y <- ArhuPCQ_WarnerRidge
#y <- ArhuPCQ_BeehiveDome
#y <- ArhuPCQ_ShinobKibe
#y <- ArhuPCQ_RedBluff
#y <- tester1
#y <- tester2
#y <- tester3
#y <- stilling.p.434
#y <- stilling.p.433

#y <- ArhuAllNested_StGeorge
y <- ArhuNest_WhiteDome
#y <- ArhuNest_SunRiver
#y <- ArhuNest_PriceHills
#y <- ArhuNest_RedWash
#y <- ArhuNest_BoomerHill
#y <- ArhuNest_WarnerRidge
#y <- ArhuNest_BeehiveDome
#y <- ArhuNest_ShinobKibe
#y <- ArhuNest_RedBluff
# observed diversity indices
# --------------------------------

S <- length(y); N <- sum(y)  # species richness and total N

rel <- y/N  # vector of relative abundances
Shannon <- abs(sum(rel*log(rel)))  # Shannon diversity
J <- Shannon/log(S)  # Shannon evenness, J

Margalef <- (S-1)/log(N)  # Margalef diversity

Simpson.D <- sum((y/N)^2)  # Simpson's D  CHECKS w/ Krebs
one.minus.Simpson <- 1-Simpson.D  # 1-D  CHECKS w/ Krebs
one.over.Simpson <- 1/Simpson.D  # 1/D  CHECKS w/ Krebs

# --------------------------------
# begin print out to R Console
# --------------------------------

dots <- ...............................................................

cat("n","n","n")  # linefeeds
cat(dots,"n","n")
cat("","OUTPUT from: "," diversity 2.R ver. Nov. 23, 2011 by 
Dwight Kincaid","n","n")
cat(dots,"n","n")
cat("","","THIS RUN: ",date(),"n","n")

cat("OBSERVED data: ",y,"n")
cat("(Abundance per taxon. Zeros not allowed here.)","n","n")
cat("OBSERVED INDICES","n","n")
cat("","Species Richness ",S,"n")
cat("","Total N ",N,"n","n")
cat("","Shannon diversity ",Shannon,"n")
cat("","Shannon evenness ",J,"n")
cat("","Margalef diversity ",Margalef,"n")
cat("","Simpson D ",Simpson.D,"n")
cat("","1-D ",one.minus.Simpson,"n")
cat("","1/D ",one.over.Simpson,"n","n",dots,"n","n")
cat("Bootstrap underway. Abundances are used to reconstitute the raw data 
which are resampled.","n")
cat("NS =",NS,"bootstrap samples. Change NS as desired. Begin low then 
increase.","n")
cat("PATIENCE, this is slow if NS is high and data set is large.","n","n")
# kincaid's bootstrap of 6 indices

# before bootstrapping, reconstitute raw data from frequency vector y

```
yy <- 1:N  # initialize vector to hold reconstituted, raw data multiplied out from the frequencies
counter <- 0; for(i in 1:S){for(j in 1:y[i]) {counter <- counter+1; yy[counter] <- i}}
```

```
resample.trees <- function(x){
  # x local var and is raw data passed from the call
  xx <- sample(x,replace=TRUE)  # bootstrap sample of raw data
  taxa <- unique(xx)  # vector of taxa in boot sample
  S <- length(taxa)  # species richness in boot sample
  N <- length(xx)  # number of individuals
  
  freq <- rep(0,S)  # vector to hold boot frequencies
  for(i in 1:S){ for(j in 1:N){ if(xx[j] == taxa[i]) freq[i] <- freq[i]+1}    }  # recover frequencies
  rel <- freq/N  # vector of relative frequencies - abundance
  
  Shannon <- abs(sum(rel*log(rel)))  # Shannon diversity
  J <- Shannon/log(S)  # Shannon evenness, J
  Margalef <- (S-1)/log(N)  # Margalef diversity
  Simpson.D <- sum((freq/N)^2)  # Simpson's D
  one.minus.Simpson <- 1-Simpson.D  # Simpson: 1-D
  one.over.Simpson <- 1/Simpson.D  # Simpson: 1/D
  
  out <- cbind(Shannon,J,Margalef,Simpson.D,one.minus.Simpson,one.over.Simpson)  # array 'out' is returned
}
```

# first we need a TIME ESTIMATE for user in case NS needs to be changed

t1 <- proc.time()  # begin bootstrap timer
boot.index <- replicate(10,resample.trees(yy))  # send the raw data; boot.indices collects Shannon
elapsed <- proc.time()-t1

cat("Elapsed minutes for a trial run of 10 bootstrap samples: ",round(elapsed[1]/60,3),"\n")
est.1 <- 10*elapsed[1]/60  ; est.1 <- round(est.1,3)  # for NS=100
est.2 <- 100*elapsed[1]/60 ; est.2 <- round(est.2,3)  # for NS=1000
est.3 <- 1e3*elapsed[1]/60 ; est.3 <- round(est.3,2)  # for NS=10,000
est.4 <- 1e4*elapsed[1]/60 ; est.4 <- round(est.4,2)  # for NS=100,000

cat("So, it will take about --",\n")
cat("      ","est.1","minutes for NS = 100 bootstrap samples","\n")
cat("      ","est.2","minutes for NS = 1,000","\n")
cat("      ","est.3","minutes for NS = 10,000","\n")
cat("      ","est.4","minutes for NS = 100,000","\n"")
cat("Hit ESCAPE to end the run, if you want to change NS in the code, otherwise be patient.")

# the full bootstrap run, by calling the above function: 'resample.trees()' # -----------------------------------------------

t1 <- proc.time()  # begin bootstrap timer

boot.index <- replicate(NS,resample.trees(yy))  # send the raw data; boot.indices collects Shannon
# boot.index[,1] is Shannon; [,2] is J; [,3] is Margalef; [,4] is D; [,5] is 1-D; [,6] is 1/D

elapsed <- proc.time()-t1  ; cat("\n")
cat("Elapsed minutes for the bootstrap: ",round(elapsed[1]/60,3),"\n","\n")

Shannon.LB <- quantile(boot.index[,1],.025); Shannon.UB <- quantile(boot.index[,1],.975)
J.LB <- quantile(boot.index[,2],.025); J.UB <- quantile(boot.index[,2],.975)
M.LB <- quantile(boot.index[,3],.025); M.UB <- quantile(boot.index[,3],.975)
S1.LB <- quantile(boot.index[,4],.025); S1.UB <- quantile(boot.index[,4],.975)
S2.LB <- quantile(boot.index[,5],.025); S2.UB <- quantile(boot.index[,5],.975)
S3.LB <- quantile(boot.index[,6],.025); S3.UB <- quantile(boot.index[,6],.975)

cat("Bootstrap 95% confidence intervals using the percentile method and achieved after NS =",NS,"\n","\n")
cat("      ","Lower","      ","Upper","\n","\n")
cat("Shannon diversity ",round(Shannon.LB,4),",",round(Shannon.UB,4),"\n")
cat("Shannon evenness ",round(J.LB,4),",",round(J.UB,4),"\n")
cat("Margalef diversity ",round(M.LB,4),",",round(M.UB,4),"\n")
cat("Simpson D ",round(S1.LB,4),",",round(S1.UB,4),"\n")
cat("1-D ",round(S2.LB,4),",",round(S2.UB,4),"\n")
cat("1/D ",round(S3.LB,4),",",round(S3.UB,4),"\n")

cat("End.","\n")

# end    diversity 2.R
Jaccard contingency table comparing presence of taxa between two sites or groups, program in R

```r
# Jaccard.R    27 March 2013  Dwight Kincaid
#
# The 2 x 2 contingency table cross-classifying the presence of taxa between
two sites or groups, etc.
#
#    site B
#    present  absent
# site A
#    present  8      4
#    absent   2      10
#
# a = 8, b = 4, c = 2, d = 10
# Jaccard = a / (a+b+c) = 8/14 = .571
# Therefore, 57.1% of the combined taxa at both sites, are present at each site.
# The sites share 57.1% of their taxa, in other words.

library(psych)

#y <- c(8,4,2,10)  # <-------- This was Kincaid's original data example
y <- c(,,,)  # <-------- enter your observed data for a,b,c,d HERE, SAVE and RUN

my.matrix <- matrix(y,nrow=2,
dimnames=list(site.A=c("present","absent"),
site.B=c("present","absent")))

print(my.matrix)

#fisher.test(my.matrix,alternative="two.sided")  # > ?fisher.test to get documentation
fisher.test(my.matrix,alternative="greater")  # or "less" for one-sided test
# note that Fisher's test 'uses' cell 'd' in the contingency table. My randomization test does not.

Jaccard <- my.matrix[1,1] / (my.matrix[1,1] + my.matrix[1,2] +
my.matrix[2,1])
print(Jaccard)

##########################################################################
#
# randomization test for comparison of observed
# Jaccard against its null distribution
#
```

# first reconstitute original data frame from the 2x2 table
N.of.total.taxa <- sum(y)
N.of.A.taxa <- my.matrix[1,1] + my.matrix[1,2]
N.of.B.taxa <- my.matrix[1,1] + my.matrix[2,1]

A.species <- 1:N.of.A.taxa     # key-off site A first
in.common.species <- 1:y[1]    # taxa as integers
B.species <- c(in.common.species, c((N.of.A.taxa + 1) : sum(y[1:3])))

site <- c(rep("A",N.of.A.taxa),rep("B",N.of.B.taxa))
species <- as.factor(c(A.species,B.species))
my.frame <- data.frame(site, species)

my.frame
table(my.frame)

# confirm that Jaccard from data frame is the same as from 2x2 contingency
table, etc.
a <- b <- c <- 0
out <- ftable(site ~ species)
table.rows <- length(out)/2

# get a,b,c
for(i in 1:table.rows) if( out[i,1] == out[i,2]) a <- a + 1
b <- sum(out[ ,1]) - a
c <- sum(out[ ,2]) - a
a / (a + b + c)   # should agree with J from original 2x2 table

# randomize reconstituted data frame in a simple loop
NS <- 1e4
null.J <- numeric(NS)    # will hold the null distribution of J
NGE <- 0

for(i in 1:NS){
a <- b <- c <- 0
null.species <- as.factor(sample(species,replace=FALSE))
null.out <- ftable(site ~ null.species)
table.rows <- length(null.out)/2

# get a,b,c
for(j in 1:table.rows) if( null.out[j,1] == null.out[j,2]) a <- a + 1
b <- sum(null.out[ ,1]) - a
c <- sum(null.out[ ,2]) - a
null.J[i] <- a / (a + b + c)
if(null.J[i] >= Jaccard) NGE <- NGE + 1
}
hist(null.J,xlab="Null Distribution of Jaccard: Arctomecon and Atriplex",
  ylab=paste("Frequency (NS =",NS,"))"),
  col="lightblue",border="white",font.lab=2,
  main="Dotted line at observed J")

abline(v=Jaccard,lty="dashed",col="red")

P <- (NGE + 1) / (NS + 1)
NGE
P

describe(null.J)

# end Jaccard.R by Dwight Kincaid
Perl script to search for repeat motif's in genetic sequences, read2Marker.pl

#!/usr/bin/perl
#-------------------read2Marker.pl-------------------
#
#           Copyright Hiroyuki Fukuoka, June 16th, 2004
#           Laboratory of Breeding Technology
#           National Institute of Vegetable and Tea Science
#           National Agriculture and Bio-oriented Research
# Organization
#           http://vegetea.naro.affrc.go.jp/

$dbRecord="../database.record";
unless (-e $dbRecord){
    open (NEWFILE, ">$dbRecord");
    close (NEWFILE);
}
open (FILE, $dbRecord);
    while (defined ($item=<FILE>)){
        chomp($item);
        push(@db, $item);
    }
close (FILE);

#make read2Marker_files directory and sub-directories (if not exist)
unless (-e '../read2Marker_files'){
    mkdir ("../read2Marker_files", 0700);
}
unless (-e '../read2Marker_files/nr_hqv_seq_files'){
    mkdir ("../read2Marker_files/nr_hqv_seq_files", 0700);
}
unless (-e '../read2Marker_files/nr_hqv_seq_files/blastdb'){
    mkdir ("../read2Marker_files/nr_hqv_seq_files/blastdb", 0700);
}
unless (-e '../read2Marker_files/nr_hqv_seq_files/blastdb/backup'){
    mkdir ("../read2Marker_files/nr_hqv_seq_files/blastdb/backup", 0700);
}
unless (-e '../read2Marker_files/moved_from_nr'){
    mkdir ("../read2Marker_files/moved_from_nr", 0700);
}
unless (-e '../read2Marker_files/fl_seq_files'){
    mkdir ("../read2Marker_files/fl_seq_files", 0700);
}
print "\n\n************ read2Marker ver. 0.9 ************\n";
print ' ' x 30, "H.Fukuoka, NIVTS\n\n";

print "data: 1) .ab1 2) .seq 3) .fasta\n";
chomp($program=<STDIN>);
if ($program==1){
  $programName="read2Marker_main.pl";
} elsif ($program==2){
  $programName="read2Marker_seqOnly.pl";
} elsif ($program==3){
  $programName="read2Marker_seqOnly.pl";
} else {
  die "bye!$!\n";
}

system("multifasta2seqfiles4r2m.pl") if ($program==3);

print "Database Name:\n";
$dbcount=0;
foreach $item(@db){
  $dbcount=$dbcount+1;
  $printstr="$dbcount") "$db[$dbcount-1]." "
  print $printstr;
  if ($dbcount > 1 and $dbcount%5==0){
    print "\n";
  }
  $dbcount=$dbcount+1;
  $printstr4new="$dbcount".) new   0) exit\n";
  print $printstr4new;

print "\ndatabase# ?  ";
for $i (0..2){
  chomp($input = <STDIN>);
  die "bye!$!\n" if ($input==0);
  if ($input=~/^[^1-9]/ or $input > $dbcount){
    die "bye!$!\n" if ($i == 2);
    print "type Database number or exit (0) ";
    exit;
  }
  last if ($input >= 1 and $input <= $dbcount);
}

if ($input == $dbcount){
  print "type new database name ? (Alphabet and numerics Only. No space, 
-, _, ..etc)\n";
  chomp($newdbName=<STDIN>);
  $newdbName=~s/\W//;
  $newdbName=~s/_//;
print "$newdbName ... ok? (y/n) ";
chomp($ans=<STDIN>);
die "bye!$!
" if ($ans ne 'y' and $ans ne 'n');
if ($ans eq 'n'){
  print "re-type new database name
";
  chomp($newdbName=<STDIN>);
  $newdbName =~ s/W//;
  $newdbName =~ s/_//;
  print "$newdbName ... ok? (y/n) ";
  chomp($ans2=<STDIN>);
  die "bye!$!
" if ($ans2 ne 'y');
}
foreach $dbname(@db){
  if(lc($dbname) eq lc($newdbName)){
    print "
***WARNING!!!***
$newdbName already exist!
"
    die "bye!$!
"  
  }
}
push (@db, $newdbName);
}

print "database = ";
print $db[$input-1];
print " ... ok? (y/n) ";
chomp ($finalans=<STDIN>);
die "bye!$!
" if ($finalans ne 'y');
if ($finalans eq 'y'){
  open (FILE,">$dbRecord" );
      print FILE join("\n", @db, "");
  close (FILE);
}

#--------Make log File
chomp($date=`date`);
chomp($libPath=`pwd`);
$libPath=$libPath./;
chdir('../');
chomp($organismPath=`pwd`);
$organismPath=$organismPath./;
chdir("$libPath" );

open (NEWFILE, '>read2Marker.log' );
print NEWFILE "Program started at ";
print NEWFILE "$date
"
$printstr="program="$programName."\ndatabase=".$db[$input-1]."\nlibPath=".$libPath."\norganismPath=".$organismPath."\n";

print "minimum query length (bp) for BLAST = ? (default=35) ";
$minLength=<STDIN>;
   chomp($minLength);
   unless($minLength>0){
      $minLength=35;
   }

print "Parameters for checking mis-priming primers:
      total length (bp) of 3'-end primer sequence to be checked ($checklen) = ? (default=12) ";
$checklen=<STDIN>;
   chomp($checklen);
   unless($checklen>0){
      $checklen=12;
   }

print "    key length (bp) to check the perfect match at 3'-end ($nn) = ? (default=3) ";
$prmkey=<STDIN>;
   chomp($prmkey);
   unless($prmkey>0){
      $prmkey=3;
   }

$taillen=$checklen-$prmkey;
$defaultmin=$taillen-1;

#print "    3'-end key length ($nn) = $prmkey";
#print "    5'-end tail length = $taillen";

die "\n[ERROR]\n $nn should be smaller than $checklen.\n" if ($taillen <= 0);

print "    minimum number of identical bases (bp) within the $taillen bp stretch adjacent to the $prmkey bp 3'-end key ($match) = ? (default=5) ";
$match=<STDIN>;
   chomp($match);
   unless($match>0){
      $match=5;
   }

die "\n[ERROR]\n $match should be between 0 and $checklen-$nn (= $taillen)\n" if ($match > $taillen);

print "    minimum number of identical bases (bp) to make a result file ($min) = ? (default=$defaultmin) ";
$min=<STDIN>;
    chomp($min);
    unless($min>0){
        $min=$defaultmin;
    }

die "\n[ERROR]\n $min should be between \$match (= $match) and \$checklen-\$nn (= $taillen).\n\n" if ($min > $taillen or $min < $match);
    print "\n\nProgram: $programName\n";
    print "\$database = $db[$input-1]\n";
    print "\$checklen = $checklen\n";
    print "\$nn = $prmkey\n";
    print "\$match = $match\n";
    print "\$min = $min\n";

$printstr="minLength_for_BLAST=".$minLength."\n";
print NEWFILE $printstr;

$printstr="keyLength_for_chkprmdup=".$prmkey."\n";
print NEWFILE $printstr;

$printstr="totalLength_for_chkprmdup=".$checklen."\n";
print NEWFILE $printstr;

$printstr="minMatch_for_chkprmdup=".$match."\n";
print NEWFILE $printstr;

$printstr="minMatch_for_result=".$min."\n";
print NEWFILE $printstr;

close (NEWFILE);

print "everything ok? (y/n) ";
chomp($ok=<STDIN>);
die if ($ok ne "y");

#--------Run read2Marker_main.pl
system ($programName);

open (FILE,">>read2Marker.log");
    chomp($date=`date`);
    print FILE "Program finished at ";
    print FILE "$date\n";
close (FILE);
Perl script for detecting and displaying variable nucleotides in aligned Fasta files, 
varSequences11.pl

#!/usr/bin/perl

### This program is free software; you can redistribute it and/or modify 
### it under the terms of the GNU General Public License as published by 
### the Free Software Foundation; either version 2 of the license, or 
### (at your option) any later version.
### This program is distributed in the hope that it will be useful, 
### but WITHOUT ANY WARRANTY; without even the implied warranty of 
### MERCHANTABILITY or FITNESS FOR A PARTICULAR PURPOSE. See the 
### GNU General Public License for more details.
### You should have received a copy of the GNU General Public License 
### along with this program; if not, write to the Free Software 
### Foundation, Inc., 51 Franklin St, Fifth Floor, Boston, MA 02110-1301 USA
### Copyright 2011 Damon P. Little

### DETERMINE OPTIONS AND ANALYSIS TYPE
my $e = -1;
my $i = 0;
my $n = 0;
my $p = 0;
my $s = -1;
for(my $k = $#ARGV; $k >= 0; $k--){
  if($ARGV[$k] eq '-e'){
    $ARGV[$k+1] =~ tr/0123456789//cd;
    if($ARGV[$k+1]){ 
      $e = $ARGV[$k+1];
    }next;
  }
  if($ARGV[$k] eq '-i'){
    if(-e $ARGV[$k+1]){
      $i = $ARGV[$k+1];
    }next;
  }
  if($ARGV[$k] eq '-n'){
    $n = 1;
    next;
  }
  if($ARGV[$k] eq '-p'){
    $p = 1;
    next;
  }
  if($ARGV[$k] eq '-s'){
    $ARGV[$k+1] =~ tr/0123456789//cd;
if($ARGV[$k+1]){
    $s = $ARGV[$k+1];
}
}

if(length($i)){
    open(INFILE, $i) || die("Could not open $i!");
    my $rawData;
    my %terminals = ();
    my $seq = ();
    my $name = ();
    my $k = 0;
    while(my $line = <INFILE>){
        chomp($line);
        if(length($line)){
            if($line =~ m/^>/){
                if(length($name) && length($seq)){
                    upload($seq, $name, $k);
                    $k++;
                }
                $seq = ();
                $name = $line;
            } else {
                $seq .= $line;
            }
        } else {
            upload($seq, $name, $k);
        }
    }
    close(INFILE);
}

sub upload {
    my $seq = $_[0];
    $seq = uc($seq);
    $seq =~ tr/ACGTNVDBHWMRKSY\-//cd;
    my @sequence = split(//, $seq);
    my $name = $_[1];
    $name =~ tr/[a-zA-Z][0-9]//cd;
    my $k = $_[2];
    for(my $j = $#sequence; $j >= 0; $j--){
        $rawData->[k]=$j+1] = $sequence[$j];
    }
    my @term = split(\_/\, $name);
    push(@{$terminals{"$term[0] $term[1]"}}, $k);
    return(0);
my @var = ();
if($s == -1){
    $s = 0;
} elsif($s > $#{$rawData->[0]}){
    $s = $#{$rawData->[0]};
}
if($e == -1){
    $e = $#{$rawData->[0]};
} elsif($e > $#{$rawData->[0]}){
    $e = $#{$rawData->[0]};
} elsif($e < $s){
    $e = $s;
}
for(my $j = $e; $j > $s; $j--){
    my %states = ();
    for(my $k = $#{$rawData}; $k >= 0; $k--){
        if($rawData->[0][$j] ne '-'){
            $states{$rawData->[0][$k][$j]} = 1;
            if(keys(%states) > 1){
                push(@var, $j);
                last;
            }
        }
    }
}

my $finalData;
my @names = sort(keys(%terminals));
my @converter = ();
$converter[0] = '-';
$converter[1] = 'A';
$converter[2] = 'C';
$converter[4] = 'G';
$converter[8] = 'T';
$converter[5] = 'R';
$converter[10] = 'Y';
$converter[6] = 'S';
$converter[9] = 'W';
$converter[12] = 'K';
$converter[3] = 'M';
$converter[14] = 'B';
$converter[13] = 'D';
$converter[7] = 'V';
$converter[15] = 'N';
    for(my $k = 0; $k <= $#names; $k++){
        $finalData->[$k][0] = $names[$k];
        for(my $j = $#var; $j >= 0; $j--){
            my $state = 0;
            for(my $i = $#{$terminals{$names[$k]}}, $i >= 0; $i--){
                if($rawData->[{$terminals{$names[$k]}}, $i][{$var}, $j] =~ m/A|R|W|M|D|H|V|N/){
                    $state = $state | 1;
                } elsif($rawData->[{$terminals{$names[$k]}}, $i][{$var}, $j] =~ m/C|Y|S|M|B|H|V|N/){
                    $state = $state | 2;
                } elsif($rawData->[{$terminals{$names[$k]}}, $i][{$var}, $j] =~ m/G|R|S|K|B|D|V|N/){
                    $state = $state | 4;
                } elsif($rawData->[{$terminals{$names[$k]}}, $i][{$var}, $j] =~ m/T|Y|W|K|B|D|H|N/){
                    $state = $state | 8;
                }
            }
            if($p && ($converter[$state] eq $finalData->[0][{$j+1}] && ($finalData->[0][{$j+1}] ne '-'))){
                $finalData->[{$k}, {$j+1}] = '.';
            } else {
                $finalData->[{$k}, {$j+1}] = $converter[$state];
            }
        }
    }

    my $max = 0;
    for(my $k = $#{$finalData}, $k >= 0; $k--){
        if(length($finalData->[{$k}, 0]) > $max){
            $max = length($finalData->[{$k}, 0]);
        }
    }
    if($n){
        $space = ' ' x ($max + 5);
    } else {
        $space = ' ' x ($max + 2);
    }

    my @thousand = ();
    my @hundred = ();
    my @ten = ();

    # PRINT PRETTY
    my $space = 

my @one = ();
for(my $k = $#var; $k >= 0; $k--){
    if($var[$k] < 10000){
        ($thousand[$k], $hundred[$k], $ten[$k], $one[$k]) =
        split(//, sprintf('%04s', $var[$k]));
    } else {
        print(STDERR "WARNING: 10,000 or more bases, script
modification is needed
"
    }
}
my $buffer = $space . join('', reverse(@thousand)) . "\n" . $space .
join('', reverse(@hundred)) . "\n" . $space . join('', reverse(@ten)) . "\n"
    . $space . join('', reverse(@one)) . "\n";

for(my $k = 0; $k <= $#{$finalData}; $k++){  
    $buffer .= sprintf('-%*s', ($max + 2), $finalData->[k][0]);
    if($n){
        $buffer .= sprintf('%2s', ($#{$terminals{$finalData-
->[k][0]}} + 1)) . " ";
    }
    for(my $j = $#{$finalData->[k]}; $j > 0; $j--){
        $buffer .= $finalData->[k][$j];
    }  
    $buffer .= "\n";
    if(length($buffer) > 10000){
        print("$buffer");
        $buffer = ();
    }
}
print("$buffer");

} else {  
    print("\nA PERL script for summarizing FASTA formated
sequences.\n")
    print("Terminals should be named
'Genus_species_[optional]'.\n")
    print("Version 1.1\n")
    print("USAGE: varSequences.pl -i in-file.fasta [-n] [-p] [-s x]
[-e y]\n");
    print("\tn-tprints number of summarized terminals\n")
    print("\tn-p\tprints periods for repeated sequence\n")
    print("\tn-s\tprints starting at position x\n")
    print("\tn-e\tprints ending at position y\n")
}
exit(0);
References


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