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Genetic Investigation Into the Diversity and Population Structure of Penstemon harringtonii (Harrington's Beardtongue)

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The Graduate School

GENETIC INVESTIGATION INTO THE DIVERSITY AND
POPULATION STRUCTURE OF *PENSTEMON
HARRINGTONII* (HARRINGTON'S
BEARDTONGUE)

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

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College of Natural and Health Sciences
School of Biological Sciences

May 2017

This Thesis by: Nathan P. Redecker

Entitled: *Genetic investigation into the diversity and population structure of Penstemon harringtonii (Harrington's beardtongue)*

has been approved as meeting the requirement for the Degree of Master of Science in College of Natural and Health Sciences in School of Biological Sciences

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ABSTRACT

Redecker, Nathan P. *Genetic investigation into the diversity and population structure of Penstemon harringtonii (Harrington's beardtongue).* Unpublished Master of Biology thesis, University of Northern Colorado, 2017.

Penstemon harringtonii is an endemic Colorado species that is listed on Bureau of Land Management (BLM) State Director's Sensitive Species List as well as on the U.S. Forest Service sensitive species list. *Penstemon harringtonii* is encountering threats from habitat destruction and fragmentation due to oil and gas exploration, livestock grazing and recreational activities. *Penstemon harringtonii* is scattered across six counties in north central Colorado. The populations split into three general areas, one around Eagle and north to Kremmling, from Glenwood Spring south to Aspen and around the community of Rifle. The disjunct nature of the species has raised questions related to the amount of genetic diversity throughout the range, population structure dynamics and rates of gene flow among populations and regions. Individuals from 20 populations of *P. harringtonii* and 6 populations of *Penstemon osterhoutii* were collected from wild populations. Additional samples of *P. osterhoutii*, *P. cyathophorus*, *P. secundiflorus*, and *P. angustiflolius* were taken from herbarium specimens or live collections in botanic gardens. Microsatellite analysis was completed using 9 variable loci to determine genetic diversity, rates of gene flow and population structure of *P. harringtonii*. Chloroplast DNA analysis was completed using three intergenic regions to determine haplotype diversity, phylogenetic relationships and patterns of maternal gene flow.

These analysis showed that *P. harringtonii* is distinct from *P. osterhoutii*. Three distinct genetic groups are present in *P. harringtonii*: Rifle, Roaring Fork River Valley and East of Glenwood Canyon. High levels genetic diversity are present with exceptional level of gene flow between genetic groups, which is great enough to maintain a cohesive species across the entire range. Inbreeding levels were low, posing minimal concern. Two population of *P. harringtonii* were found to be quite distinct at the northern and southern extents of the population when compared to the region genetic groups. Conservation and land management agencies now have genetic information that can be utilized to inform decisions about the conservations of *P. harringtonii*.

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Finally, I would like to extend a big thank you to all friends and colleagues at the University of Northern Colorado and elsewhere. Additionally, extend a big thank you to my parents Dan and Cindy Redecker for providing emotional support and encouragement throughout all of my educational endeavors.

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OVERVIEW OF *PENSTEMON HARRINGTONII*,
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Introduction

This research investigates the variation in microsatellite loci and selected

chloroplast sequences for a rare Colorado endemic wildflower species. *Penstemon*

harringtonii Penland (Plantaginaceae) inhabits a limited range in western and central

Colorado around the I-70 corridor. This *Penstemon* is found in open sagebrush and less

often in pinyon-juniper from 2000-2800 meters (6800-9200 feet). The range of the

species is on public lands for the majority of known occurrences, which provides

potential protections, but also introduces additional threats that may be detrimental to the

species. This project was tasked with determining the genetic relationship between

populations of *P. harringtonii* throughout its range in Colorado. Increases in oil and gas

extraction in the western part of the range, small mining operations and grazing

throughout the range, and residential development in the Eagle River valley has increased

pressure on this species. Due to these disturbances, understanding how the current

populations are interacting in terms of genetic connectivity is critical to ensure that

appropriate management decision are made to maintain the species presence on the

landscape. This chapter addresses 1) Genus *Penstemon* Schmidel (Plantaginaceae), and

P. harringtonii description and life history; 2) overview of the threats and to what degree

they may affect *P. harringtonii* throughout its range; 3) land management objectives and actions that are being implemented to aid in the persistence of rare and endangered species; 4) description of the aims and methods of this project to establish context for the following chapters.

Penstemon **Genus**

Penstemon is a well-known genus that was first described by Mitchell in 1748 and then by Schmidel in 1763 that consists of over 270 species, mainly concentrated in North America (Straw 1966; Nold 1999). Until recently, *Penstemon* had been in the Scrophulariaceae family, which was often thought of as an inconsistent family due to an incoherent set of diagnostic characteristics. With advances in molecular techniques, Scrophulariaceae has been reduced in size and numerous genera have been filed into other families, including *Penstemon,* which is now in the Plantaginaceae family (Albach et al. 2005). *Penstemon* makes up the largest assemblages of species within Plantaginaceae that are endemic to North America, with a significant portion residing solely in western North America. *Penstemon* is a representation of a continental evolutionary radiation driven by pollinator adaptations, allowing numerous species to coexist in relatively small areas (Straw 1966; Wolfe et al. 2002; Wolfe et al. 2006). From this adaptive radiation, numerous species have emerged that are endemic to a single state or a single region, leading to an increased extinction risk due to rarity (Straw 1966; Wolfe et al. 2006). The genus is classified into six subgenera, with two being monotypic and the other four being separated based on morphological traits such as habit, flower structure, and leaf and stem characteristics (Wolfe et al. 2006). *Penstemon harringtonii* is in subgenus *Penstemon* which includes species with non-woolly anthers opening end to

end, and subsection *Coerulei* which includes species with an herbaceous woody base, thick and leathery leaves, tubular corolla and a staminode bearded with golden hairs (Penland 1958; Nold 1999; Wolfe et al. 2006).

Penstemon harringtonii

Morphology. *Penstemon harringtonii* is a herbaceous perennial plant with one to two stems; hairless, leathery, and entire leaves that are oblanceolate to spatulate in shape. Flowers are light purple to blue, sometimes with pink at the edge of the floral tube. The floral tube is well developed and distinctly bilabiate. One distinctly pubescent staminode is present along with, four didynamous stamens, two of which are well exserted from the edge of the floral tube (Penland 1958). Currently, *P. harringtonii* has protection under the Bureau of Land Management (BLM) State Director's Sensitive Species List as well as the United States Forest Service (USFS) (Region 2) sensitive species list. This species was first described by Penland in 1958 from a site in Grand County, Colorado northwest of Green Mountain dam. The two exserted stamens are the most effective character used to distinguish it from closely related species that share similar vegetative morphology. *Penstemon osterhoutii* Pennell is sympatric and has similar flora coloration, overall habit, and leaf texture and shape, but lacking the two exserted stamens and tending to be larger in size. When flowers are present *P. harringtonii* and *P. osterhoutii* can easily be distinguished (Penland 1958; Panjabi and Anderson 2006). *Penstemon cyathophorus* Rydb. is believed to be the closest relative to *P. harringtonii (*Wolfe et al. 2006; Wessinger et al. 2016), with a sympatric distribution in the northern extents of *P. harringtonii's* range. *Penstemon cyathophorus* is distinguished due to the four exserted stamens, and morphology of these two are similar but easily identified from one another

even without flora structures available (Penland 1958). *P. harringtonii* has loose inflorescences, strongly reduced bracts that are mostly longer than broad and anther 2.5-3 mm long; while *P. cyathophorus* has dense inflorescences, prominent bracts that are mostly broad than long and anther 1.2-2 mm long.

      Habitat. *Penstemon harringtonii* is found in open sagebrush of the intermountain region of Northwest Colorado along the upper Colorado River, Eagle River and throughout the Roaring Fork River Valley (RFRV) (Figure 1). There are three areas of concentration based on currently known occurrences: 1) the upper Colorado River, east of Glenwood Canyon, north to Kremmling and along with Eagle River west of Vail; 2) the Roaring Fork River Valley from Glenwood Spring to just west of Aspen; 3) to the south of the city of Rifle. These three areas all have a combination of open sagebrush with some areas having varying degrees of pinyon-juniper. Soil types where *P. harringtonii* is found are some combination of loam or clay-loam of calcareous parent material with unknown reliance on unique or specific substrates (Panjabi and Anderson 2006).
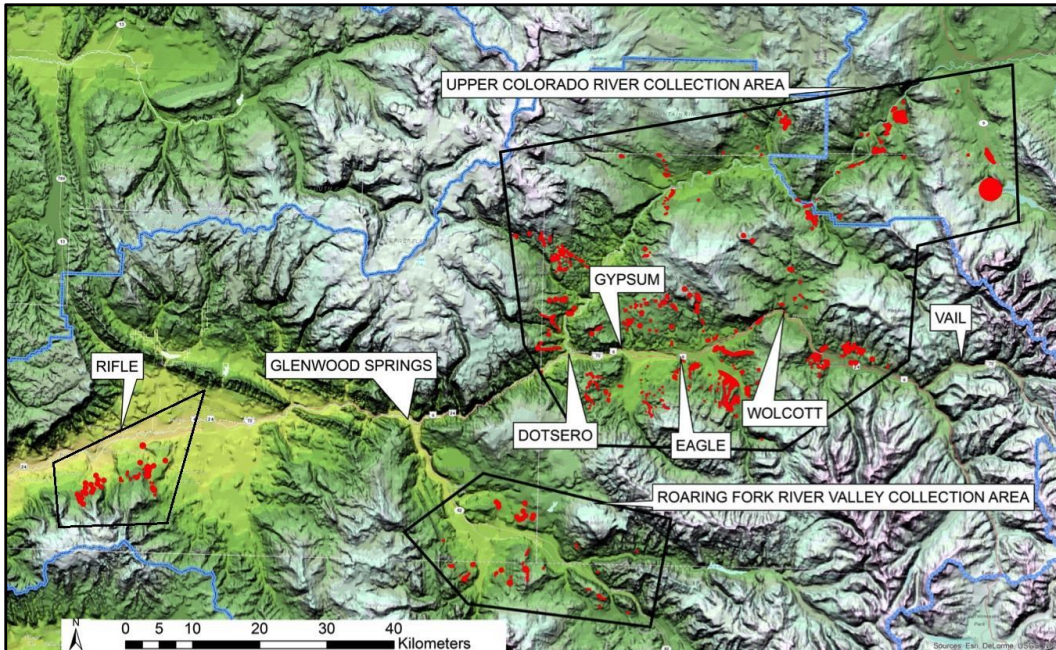
Figure 1. Current occurrence records for *Penstemon harringtonii* across its range from the Bureau of Land Management, Colorado Natural Heritage Program and personal observation.

Life history. *Penstemon harringtonii* is a perennial that has inconsistent flowering from year to year, which is thought to be related to precipitation amounts, but without site based weather data these conclusions are hard to validate (Panjabi and Anderson 2006). In the field, observations of individual's size fall into the range of the initial species description, but variability is still prevalent with some sites displaying stunted and overly large individuals. Individuals are self-compatible but are naturally cross-pollinated by numerous species of bees in the Megachilidae family, wasps of the Subfamily Masarinae and bee-flies of the Bombyliidae (Crosswhite and Crosswhite 1966; Nielson 1998; Panjabi and Anderson 2006). The dominant pollinators for *P. harringtonii* are Megachilidae bees in the genus *Osmia* (Nielson 1998), but variability has been observed from year to year and site to site, with one central pollinator not being seen at a consistent frequency (Crosswhite and Crosswhite 1966). The variability seen in pollinator visitation indicates that pollinators are not a restricting factor for this species.

Flowering occurs in June and July with fruit maturation in August and September

(Penland 1958; Nielson 1998; Panjabi and Anderson 2006). Fruit set is consistent across

the range and elevation gradient, with no significant differences observed (Nielson 1998).

Seed production is consistent across the range with a slight, non-significant decrease with

an increase in elevation, therefore seed production is not seen as a restricting factor either

(Nielson 1998). Seed germination is a critical variable that has not been investigated well

enough to establish a rate of germination from year to year or to compare between

differing precipitation years. Seed germination is a critical piece of information needed

to fully understand the life history of plants, especially rare species (Schemske et al.

1994). Seedlings, juveniles, non-flowering adults and flowering adults are seen at sites,

indicating that these stages are occurring at a regular rate, but herbivory, where present,

may be depressing successful completion of the life cycle (Schemske et al. 1994; Panjabi

and Anderson 2006; Grant III et al. 2012; Hufft and DepPrenger -Levin 2015).

Conservation and Management

Penstemon harringtonii is a species endemic to Colorado that currently has

protection under the Colorado BLM State Director's Sensitive Species List as well as the

USFS Region 2 Forester's Sensitive Plant species list. On BLM-administered lands,

BLM will manage Bureau sensitive species and their habitats to minimize or eliminate

threats affecting the status of the species or to improve the condition of the species

habitat. When a project potentially impacts a population, the appropriate National

Environmental Policy Act (NEPA) process begins, to analyze the potential effects on the

species to stay in accordance with Forest Service Manual 2670.32 and BLM Manual

Transmittal Sheet 6840.2B, both outlining guidelines for managing special status species
(USFS 2005; BLM 2008).

Currently, USFS is not doing any kind of intensive monitoring of this species.
The monitoring of *P. harringtonii* on BLM lands is being conducted by the Denver
Botanic Gardens (DBG) and the BLM. In 1996, Denver Botanic Gardens and the Bureau
of Land Management initiated a long-term demographic study of *P. harringtonii* to
quantify population fluctuations within two of the 44 known populations of the species.
The plots are located in the Eagle River Valley near the town of Eagle and near Gypsum
at Dry Lake. Data collected from these plots is used to monitor the overall trend at these
two sites and to correlate temperature and precipitation to reproductive output (seedling
density), plant vigor, plant density, flower count, and herbivory (Hufft and DepPrenger -
Levin 2015). In addition to the work by DBG, the BLM Colorado State Office uses
"point in time" sampling (Sample size equation #1,Elzinga et al. 1998) to estimate mean
density and population size at specified locations throughout the range of this species to
supplement the long-term trend monitoring data (Dawson 2015). Current management of
this species is following the guidelines as outlined in FSM 2670.32 (USFS 2005) and
BLM MTS 6840.2B (BLM 2008). Impacts to *P. harringtonii* and its habitat are
addressed in land use plans and associated NEPA documents. Molecular data for *P.
harringtonii* across its range will provide BLM with additional information to develop
proactive conservation strategies that reduce or eliminate threats to the species at the
appropriate spatial scale (BLM MTS 6840.2C). Delineation of the species would include
identification of genetically distinct groups that show evidence of independent
evolutionary changes that indicate limited interaction between areas. Monitoring

populations and habitats of rare plants, especially within potentially high disturbance areas, is a goal of the Special Status Species Management (6840 Manual) of BLM, and understanding the relationship between the subpopulations of *P. harringtonii* will allow management to more effectively monitor the species. Documentation of genetically distinct groups will enable the BLM to develop a conservation strategy for this species to potentially minimize the need for listing under the ESA.

Population Structure

The genetic relationship between inhabited regions of the species is still unknown. The distribution of these regions are disjunct. Currently, the area between the Eagle-Gypsum and the Northern Colorado River (NCORV) is lacking populations that would allow for significant gene flow between the two areas (Figure 1). The Roaring Fork River Valley (RFRV) populations are effectively isolated from the Gypsum-Eagle population by the northern edge of the Sawatch Mountains and the Southern extremes of the Flat Tops Mountains and thought to be isolated from the Rifle populations, supported by the lack of individuals found between the two sites. Most of the populations that are mapped occur on BLM lands. In cooperation with the BLM, the current rare plant management plan would be adapted to include additional objectives to implement protections for the populations that were shown to be critical for the species persistence based on the molecular data. Protection of the actual plants is crucial, especially if the populations' range is split into distinct genetic clusters that might need special protection to ensure that the distinctiveness that designated the clustering persists on the landscape. BLM will manage this species according to the Land Use Plan of the Field Office and may develop a species-specific conservation strategy for this species on BLM-

administered lands. These molecular data can provide justification for removal from the sensitive species list for Colorado.

Seed Collection

Seed collections should be made to maintain genetic material in storage from each distinct region across the range of the species along with voucher specimens. Seed collections for *P. harringtonii* would require a collection permit from the land managing agencies from which the collections are taken. These collections will provide the necessary genetic material to propagate needed seed that will be planted as deemed necessary to ensure the survival and persistence of the species across its range. Where heavy disturbance is thought to have resulted in extirpation of the species from an area, in-depth assessment and the reduction of the disturbance forces would need to take place before seeding of any kind was allowed, to ensure the likelihood of re-establishment was high enough to pursue the restoration option. A seed collection strategy should capture as much of the genetic diversity that gives a representation of populations from throughout the range of the species (Falk and Holsinger 1991; Guerrant et al. 2014). Falk and Holsinger (1991) recommend that if a rare species has five or move extant populations sampling from five of all populations will capture the majority of the genetic diversity at the population level, but more populations should be sampled if low gene flow between populations is occurring. As for how many individuals need to be sampled, Brown and Marshall (1995) state that 30 plants are needed to capture 95% of the genetic representation within a population of a completely outcrossing sexual species or 59 plants from a population of completely self-fertilizing species. Crossa and Vencovsky (2011), recommend collecting from 187 to 172 plants based on probability models that look at

theoretical allele frequency at a subset of alleles. The most recent investigation into how much sampling is required to ensure that common alleles are captured through collected materials concluded fewer samples are needed. McGlaughlin et al. (2015) indicated that sampling 10-30 individuals captured 90% of the wild genetic diversity in a rare annual plant as seen in observed heterozygosity (H_O), expected heterozygosity (H_E) and effective number of alleles (N_E). This study will give land managers that needed information to target the appropriate populations for seed collections.

Genetic Understanding

Understanding the genetic makeup of individual populations is key to understanding the direction the species is going in terms of its evolutionary journey. With species that have low genetic diversity and population numbers, extinction is more likely to occur (Schemske et al. 1994). Low genetic diversity can be caused by a lack of input of mutations, low genetic drift rates, reduced gene flow, detrimental selection events, and population bottlenecks, which result in species with minimal ability to respond to change because of the limited genes available to be expressed or recombined (Freeland et al. 2011). High genetic diversity across a range would indicate stability of the species and needed action is limited. Genetic diversity within a population allows the species to adapt to changing environmental forces because numerous different genotypes are present (Agashe 2009). The in-population heterogeneity will reduce the overall extinction risk of the species, just as management focused on increasing the diversity in populations is better to reduce extinction risk than focusing solely on population size (Fox 2005).

Recent molecular investigations have determined that *Penstemon* is a part of Plantaginaceae family (Albach et al. 2005). In addition, the complex genus has long been looked at to try and determine the appropriate classification of species in the numerous tribes, subgenera and sections that make up the *Penstemon* (Penland 1958; Straw 1966; Nold 1999; Wolfe et al. 2002; Wolfe et al. 2006; Dockter et al. 2013; Wessinger et al. 2016). Wessinger et al. (2016) documented variability among previously diagnosed clades, which is partially due to a combination of Bayesian methods, small sample sizes and method of data collection to determine how certain section coalesced. This study may not contribute significantly to the understanding of the evolution of the genus as a whole, but will contribute to the understanding of a single species phylogenetic location, the prevalence of recent adaptive radiation events that resulted in the diversity in *Penstemon*, the relationship between closely related species, and how true geographic and/or pseudo-geographic barriers affect *P. harringtonii* population dynamics across its range. Speciation within species that have disjunct populations can occur due to the historical fragmentation or dispersal and resulting founder events (Orellana et al. 2009). The potential for the discovery of genetic distinctiveness between populations of *Penstemon harringtonii* could result in an incipient speciation event. Understanding and defining speciation events, especially incipient events is difficult because of the lack of a universally excepted model for the process and definition of speciation (Coyne and Orr 2004). Due to the difficulty of tracking and defining speciation events, management agencies should manage at the population level to maintain the species as originally described.

Threat Assessment

Proactive management that follows best management practices for the species and adheres to the land use plans, mitigates the detrimental effects that might occur within the range of the species. Mitigation of threats will reduce the chance of population bottlenecks therefore maintaining genetic diversity, or at the very least reduce the degree of a bottleneck event by preventing a larger portions of the population from being removed. In addition, mitigating threats can ensure that population connectivity is maintained throughout the species' range. In addition to *in situ* management, *ex-situ* practices are an important component of the management of this species. Seed collections representing the genetic variation of the species throughout its range and the maintenance of these collections are essential. When *in situ* practices are insufficient to maintain gene flow, management can utilize seed collections as needed to mitigate poor performing populations. Speciation and extinction events should not be the indicators for management to take action. Management agencies should proactively manage to minimize or eliminate threats that affect the status of this species.

In the western United States, *Penstemon* is a very diverse group and could potentially be a key resource for pollinators throughout the landscape. No current studies show that *P. harringtonii* has any unique relationship with a specific genus or species of pollinator. Studies have shown that pollinator groups can have a higher affinity for certain families or genera, but still utilize other flora (Crosswhite and Crosswhite 1966). In addition to pollinator affinity for one species or another, a general decline in pollinators across North America and Europe is thought to be a combination of environmental stressors, pathogens/pests, and genetic variability issues of the species

(Crosswhite and Crosswhite 1966; Potts et al. 2010). Maintaining persistent, healthy and stable populations of attractive pollen and nectar sources on the landscape, like *P. harringtonii*, allow for that landscape to support more pollinators. Even though no significant effects have been found to support that the decline of pollinators is a detriment in the area where the species is found, pollinators are key to the long-term persistence of species. This study is not including a pollinator component, but pollinators are vital to maintaining genetic diversity in outcrossing insect pollinated flora (Clare et al. 2013). Further pollinator research with *P. harringtonii* is warranted.

Geographic Threats

The three geographic areas where this plant is found vary in habitat quality and composition, which could impact the current survival and long-term persistence of the species across its range. The Eagle and upper Colorado River area is the largest of the areas, covering from the relatively populated I-70 corridor to the sparsely populated sage-steppe landscape along the Colorado River Byway that leads to more populated areas near Kremmling, CO, the northernmost extent of this species' range. The Roaring Fork River Valley (RFRV) is the second area of interest for this species. The Roaring Fork River splits the Sawatch Range to the east and the Elk Mountains to the west; populations are scattered at various elevations and vegetation types throughout the foothills of these mountain ranges. *Penstemon harringtonii* is found on mountain top meadows where no shrub component is present to hillsides and hilltops that range from sagebrush, sage-juniper to pinyon-juniper habitat types. The populations are scattered and relatively distant from large urban centers where the majority of known populations are found on BLM and USFS lands. The area around the community of Rifle is the third area of

habitat for *P. harringtonii* and represents the westernmost extent of its range.

Populations are found at higher elevation plant communities to the south of Rifle on

BLM lands. Sage-steppe is the major vegetation class in the area with a small percentage

component of *Juniper* sp. present.

Threats and Public Lands

Public lands are the focus for federal and state resource management agencies,

which are tasked with balancing the needs of a diverse community of stakeholders.

Human population levels are increasing in these mountain communities and with that,

construction is occurring in places that were once viewed as not ideal building sites.

Zoning and planning regulations put forth by the County Board of Commissioners do

provide some land use restrictions for seasonal wildlife and their associated habitat,

wildfire mitigation, hillside and ridgeline development and unstable or fragile geologic

sites (Eagle County Land Use Regulations, Chapter 2, Article 4, 2015). These types of

regulations provide a broad scale protection of potential *P. harringtonii* habitat and are

better than nothing. This study will focus on the public lands and the threats that are

associated with habitats of each aforementioned region.

The species is found predominately on BLM lands with a small portion being

identified on USFS and State lands. Land management across these areas vary, but do

adhere to a multiple use type management that can result in varied disturbance regimes.

The following is a summarization of the primary disturbance types that are present

throughout the range of the species. Grazing is present across all regions and depending

on if animals are on a parcel or not, animal density and duration of utilization will

determine the extent of the disturbance in the area. Oil and gas development is the other

major disturbance factor that is present on public lands. The development of well pads

and roads destroys potential habitat and can have other indirect detrimental effects on the

species. Increased vehicle usage in these areas results in the potential for the introduction

of invasive species at the edges of the roads and well pads. Other land uses include

recreation such as mountain biking, off-road vehicle use, camping, and hiking which all

have a limited effect on the surrounding habitat if adherence to trail signage and

backcountry etiquette occurs. These recreational activities become a threat when users

don't adhere to rules and regulations in place to protect important habitat and sensitive

species. Finally, the general infrastructure that is put in place by agencies to effectively

manage are conduits for invasive species and another incidence of habitat fragmentation.

Increasing awareness for public land users about rare/sensitive plant habitat and how their

actions on public lands might impact the species and providing information to advise

alternative behavior and build awareness could potentially mitigate a portion of these

disturbances events in areas of concern. This species has many different disturbance

events to contend with which could result in loss of populations and cohesiveness

throughout the species, potentially resulting in divergence. If unique genetic clusters

have developed in certain areas of the species' range, proactive management decisions

could be made where the BLM can develop a series of conservation agreements to aid the

overarching habitat conservation objectives of the land use plans. In addition to a

conservation agreement, this study will put forth a set of best management practices for

Penstemon harringtonii for the BLM and other land managing agencies to incorporate

into their management.

Study Aims and Methods

The following chapters will outline the procedures and outcomes of the genetic

analysis of *Penstemon harringtonii*. The aim of this study is to determine the genetic

relationships between the three disjunct *P. harringtonii* regions through the utilization of

chloroplast Deoxyribonucleic acid (DNA) and nuclear microsatellites. Genetic

relationships will be determined for all inhabited regions, populations that reside in close

proximity and dynamics within populations. Genetic diversity will be determined

between and among populations across the range of the species to determine the viability

of the species as a whole. From the collected data an understanding of the population

structure will be determined, from which conservation and management decisions can be

better informed to ensure that public and private land agencies have the best available

science to make their management decisions. Chapter II is an overview of microsatellite

data collection and analysis. Chapter III is an overview of chloroplast data collection and

analysis. Chapter IV summarizes all of the findings from chapter II and III, applying

them to land management in a series of recommendations for land management agencies

that operate throughout the range of the species *P. harringtonii*.

CHAPTER II

MICROSATELLITE ANALYSIS

Introduction

Habitat modification is prevalent throughout the western United States, with

grazing, recreation, and oil and gas exploration being some of the most notable sources.

With most anthropogenic activities, infrastructure is necessary for the activity to occur

and continue, which results in habitat loss and fragmentation. Understanding the effects

of anthropogenic activity on native organisms is critical to managing the lands

appropriately, so managers can effectively determine how fragmentation is impacting

plants in order to determine necessary conservation and management actions. Whether

habitat fragmentation has detrimental effects on native organisms is hard to quantify for

some (Hadley and Betts 2012) and straightforward for others (Olivieri et al. 2008; Hale et

al. 2013). If the disturbance is large enough, in effect removing the majority of usable

habitat from an area, the native organisms will no longer be present because of a lack of

suitable habitat. In addition to the density of the disturbance, an increase in vectors to

move organisms in and out of an area may lead to invasive organisms becoming

prevalent (Manier et al. 2014) and outcompete native indivuiduals or populations

resulting in a decline and potential extripation. These vector could also manipulate the

movement of genetic material across the landscape; gene flow. A shift in gene flow

between native populations, whether that's a decrease (Hale et al. 2013) effectively

isolating individuals or population resulting potential differeniation and divergenece, or

increase (Zarlenga et al. 2014) which maintains necessary exchange of genetic material for a species to remain cohesive. Generally, species that experience habitat fragmentation in their range will result in segmented populations with reduced gene flow. This isolation can lead to gradual genetic drift within groups, leading to some differentiation between populations (Freeland et al. 2011; Spurgin et al 2014). Without the influx of new genetic diversity via gene flow, the rate of inbreeding will increase resulting in detrimental inbreeding depression (Freeland et al. 2011). In addition to the loss of genetic diversity, pollinators can be affected by fragmentation through the loss of habitat, nectar and pollen sources (Hadley and Betts 2012), and with a loss of pollinator functionality fragmentation will have a greater affect, even at a small scale. Microsatellite analysis will provide a better understanding of the relationship between populations and how genetic material is being moved across the landscape.
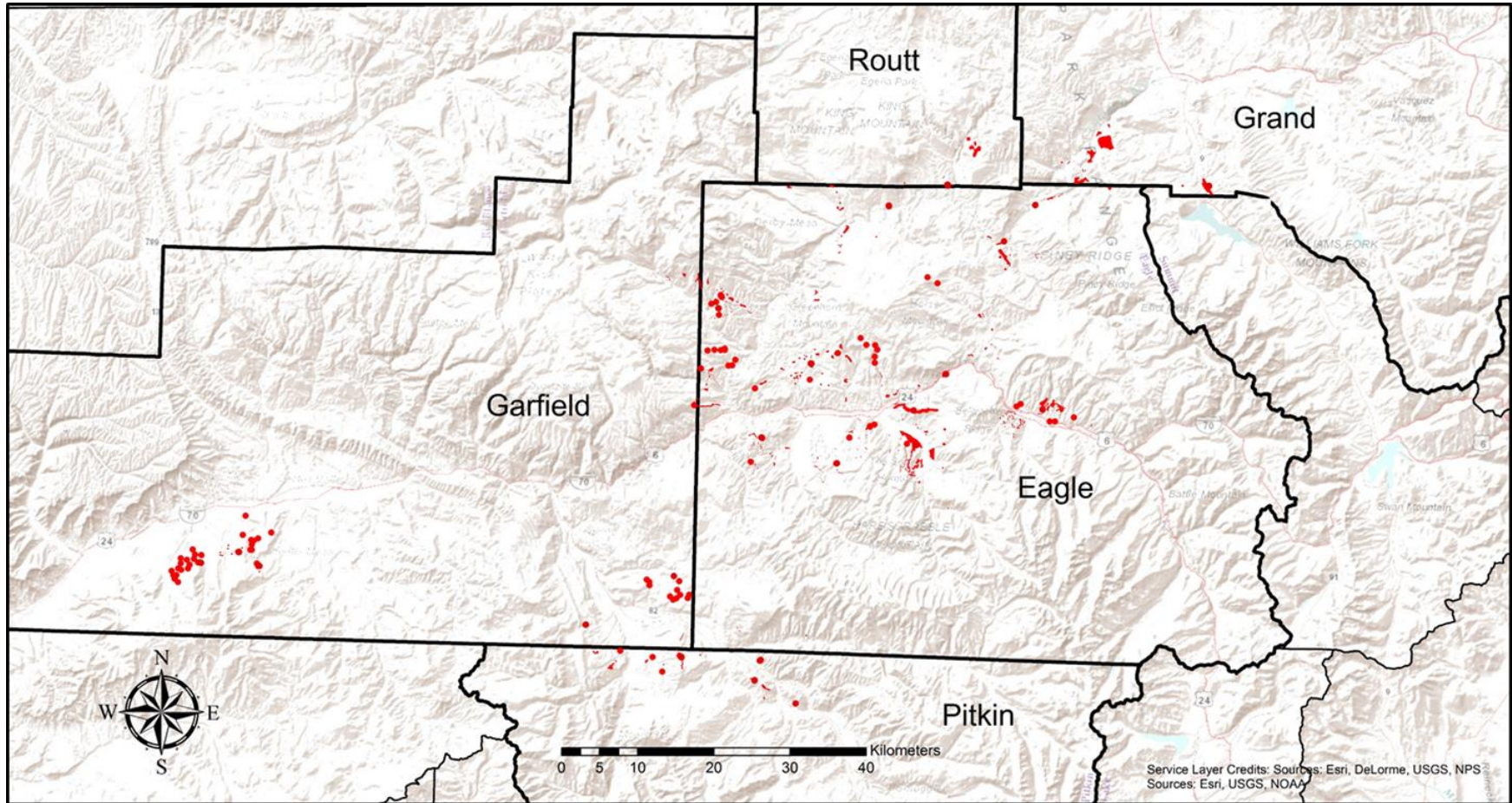
Figure 2. Population distribution map for *Penstemon harringtonii*.
Red points indicates areas where *P. harringtonii* was found in the past 10-15 years.   All data is the consolidated from the Bureau of Land Management, Colorado Natural Heritage Program, Denver Botanic Gardens and personal observations.

Three main groups of *Penstemon harringtonii* individuals have been identified, Rifle, Roaring Fork River Valley and the Eagle areas. These groups are isolated from each other by 15 to 40 miles. The range of the species is restricted to Grand, Eagle, Pitkin and Garfield counties (Figure 2). Within these counties, *P. harringtonii* is facing increasing levels of habitat disturbance and modification. Oil and gas related disturbance dominates central Garfield County, while recreation and grazing are the dominant sources of disturbance in Grand, Eagle, and Pitkin counties, with the disturbance pressure varying by specific location. Through these activities, habitat and populations are being disturbed and destroyed, and new barriers to gene flow may be created, effectively isolating populations, or new corridors of gene flow may be formed due to increased anthropogenic traffic between areas.

A genetic investigation is necessary to determine the relationship between *Penstemon harringtonii* populations throughout its range to determine present levels of genetic diversity, rates of gene flow, and overall population structure. This investigation will determine genetic diversity by measuring heterozygosity levels and allelic diversity, gene flow through number of migrants and network connectivity and determine population structure by STRUCTURE software and Phylogenetics. Understanding the population structure of this species will help land management agencies effectively select appropriate populations for conservation efforts. Resulting genetic structure will indicate levels of differentiation, gene flow will determine levels of admixture and overall cohesiveness of the species between regions. Previous genetic studies into several *Penstemon* species have recommended conservation actions that would result in necessary protections of the focal species. Wolfe et al. (2014) looked at *Penstemon*

debilis across its fragmented range and conclude that habitat conservation was the best course of action to maintain population integrity and species continuity. Kramer et al. (2011) looked at three *Penstemon* species with three pollinator syndromes to identify how landscape affected the genetic structure across populations. Kramer et al. (2011) were able to determine the type of pollinators that were best at maintaining higher levels of cohesion within species, resulting in less structure across the landscape, and other types of pollinators that were ineffective, which resulted in genetic structure based on perceived geographic barriers. Flying insect versus bird pollinators is the determining factor for measuring the degree of effect on genetic structure. Insects effectiveness will be based on body size, as it increases less structure is present because travel distances are greater, birds are the same but significantly more which allows for more admixture between populations and overall less structure as the result. Pollinator related genetic structure provides an additional aspect to consider to effectively incorporate pollinator affects into the management of species (Kramer et al. 2011). Johnson et al (2016) utilized molecular techniques to effectively re-identify herbarium specimens and field samples of *Penstemon luculentus* to correct the record for the range, and ensure that land managers are surveying in the correct areas. Utilization of genetic data can effectively provide support for population level questions of unique species and identify how landscape level factors are affecting the same or similar species, which can be used to successfully manage the species as the landscape changes or shifts.

Penstemon harringtonii was classified as a new species based on morphological characters as outlined by Penland (1958). Penland (1958) indicated that *P. harringtonii* most resembled *P. osterhoutii* overall habit, but they were differentiated by anther sac

shape, sagittate versus divaricate, and two exserted stamens in *P. harringtonii* and no stamen exsertion in *P. osterhoutii*. Due to the largely overlapping range of *P. harringtonii* and *P. osterhoutii*, there is concern that *P. harringtonii* may be recognized based on plastic traits or be a regional variant. *Penstemon osterhoutii* is a more widespread species the stamen morphology being the character that allows the two species to be distinguished from one another (Penland, 1958). Without stamen, vegetative characteristics are similar enough for field identification to be difficult and unreliable (Personal Observations; Panjabi and Anderson, 2006). Wolfe et al. (2002; 2006) used the chloroplast matk gene and non-coding regions *trnC-D* and *trnT-L,* and nuclear rDNA ITS, intergenic internal transcribed spacer, sequences to construct phylogenies for *Penstemon*. Though Wolfe et al. (2006) utilized chloroplast and nuclear data, more samples from a wider range of *Penstemon* species resulted in limited resolution of the relationships between species. These unresolved relationships confirms that many species of *Penstemon*, including *P. harringtonii*, lack explicitly defined lineages. Unresolved relationships within *Penstemon* are likely a result of the recent radiation of the genus (Wolfe et al. 2002; 2006)*,* and due to this rapid diversification of species. Understanding and quantifying the amount of gene flow, diversity and population structure of *P. harringtonii* as well as assessing levels of admixture with *P. osterhoutii* will support the separation of the species and determine the status of *P. harringtonii*.

Penstemon harringtonii* has had minimal pollinator specific investigations completed, but other *Penstemon* species have been investigated. A Master's thesis looking into the reproductive biology and ecology of *P. harringtonii* completed by

Neilson (1998). Pollinators specified for *P. harringtonii* based on visitation frequency were bees of the family Megachilidae and wasp of the family Vespidae, subfamily Masarinae (Neilson 1998). Pollinators are critical to the exchange of genetic material across a landscape and depending on the mobility of pollinators will result in various degrees of population structure (Bustamante et al. 2016; Breed et al. 2015; Pasquet et al. 2008). The disjunct populations of *P. harringtonii* create an uncertainty of the level of gene flow between groups of populations and depending on pollinators may result in differentiation. Kramer et al. (2011) determined that for three common *Penstemon* species different pollination syndromes determined the overall genetic structure across the range of a species. Kramer et al. (2011) found that bird pollination results in reduced genetic structure to almost no structure with high amounts of genetic admixture between populations. Different sized bees resulted in various degrees of genetic structure, with the range of travel for bees determining the amount of structure within *Penstemon* populations (Kramer et al. 2011). Bees with large foraging ranges resulted in established structure based more on geographic barriers while bees with smaller ranges result in structure associated with local populations. Current knowledge on *P. harringtonii* is that they are pollinated by medium sized bees with large foraging ranges but are not solely dependent on a specific group of pollinators (Neilson 1998). The disjunct nature of *P. harringtonii* populations indicates that significant structure should be present with differentiation between regions separated by geographic barriers. Based on floral tube structure some bird pollination may be occurring but the Neilson (1998) investigation didn't observe any.

In this chapter, nine polymorphic nuclear microsatellite markers are examined to determine population genetic structure within and among populations of *P. harringtonii*. Through the analysis of microsatellites, recent patterns of gene flow, genetic diversity, and the relationship between *P. osterhoutii* and *P. harringtonii* were examined. The goal of this study is to inform conservation and land managing agencies if 1) *P. harringtonii* exhibits distinct genetic structure, 2) determine the relative gene flow between and among populations, 3) determine levels of genetic diversity and inbreeding, and 4) determine the relationship between *P. harringtonii* and *P. osterhoutii*. Conservation recommendations will be developed from the data in this chapter that will provide information to land managers.

Methods

Population Sampling

Collection sites were scouted by Bureau of Land Management personnel from the Colorado River Valley Field Office in Silt, CO. In addition to pre-scouted locations, element occurrence records were utilized to find additional locations to fill in gaps within the range of the species. Two periods of collection took place, one during the summer of 2015 and the other during the summer of 2016. Overall, 18 *P. harringtonii* populations and six populations of *P. osterhoutii* were sampled (Figure 3, Table 1). Four populations were initially collected as *P. harringtonii*, McCoy, Catamount, Barber's Gulch and Wingo Junction, but based on data analysis in this chapter and the next, they were concluded to be *P. osterhoutii*. One of the populations, McCoy, was found to contain both *P. harringtonii* and *P. osterhoutii* individuals. Sampling consisted of collecting one or two basal or cauline leaves from a target of 32 individuals, or as many individuals as

were observed at a site. The tissue samples were placed in individually labeled bags and

put on ice until they could be stored in a freezer. GPS coordinates were taken for each

individual collected.

Table 1. The populations used in this study with the species name, population identifier, population name, Colorado county and region where the population is located, and number of individuals collected from each population.

Species and Population ID	Population	County	Region	*N*
P. harringtonii				
AG	Agnew Gulch	Eagle	Eagle	32
BC	Berry Creek	Eagle	Eagle	32
EE	East Eagle	Eagle	Eagle	32
MG	Mayer Gulch	Eagle	Eagle	32
NH	North Hardscrabble	Eagle	Eagle	32
OR	Onion Ridge	Eagle	Eagle	32
SCU	Sheep Creek Uplands	Eagle	Eagle	32
RC	Red Canyon	Eagle	Eagle	32
RH	Red Hill	Eagle	Eagle	32
MC	McCoy	Eagle	NCORV	12
CH	CO10H9	Grand	NCORV	33
SB	State Bridge	Eagle	NCORV	32
YM	Yarmony	Eagle	NCORV	32
LH	Light Hill	Pitkin	RFRV	32
CR	Crown	Pitkin	RFRV	32
WH	Williams Hill	Pitkin	RFRV	32
CC	Cattle Creek Rd	Garfield	RFRV	6
SG	Spruce Gulch	Garfield	Rifle	32
GM	Grass Mesa	Garfield	Rifle	32
FIM	Flat Iron Mesa	Garfield	Rifle	32
P. osterhoutii				
PC	Prince Creek	Garfield/Pitkin	RFRV	10
AP	Anvil Points	Garfield	Rifle	10
MC	McCoy	Eagle	NCORV	20
CM	Catamount	Eagle	NCORV	32
WJ	Wingo Junction	Pitkin	RFRV	32
BG	Barber's Gulch	Garfield	RFRV	32
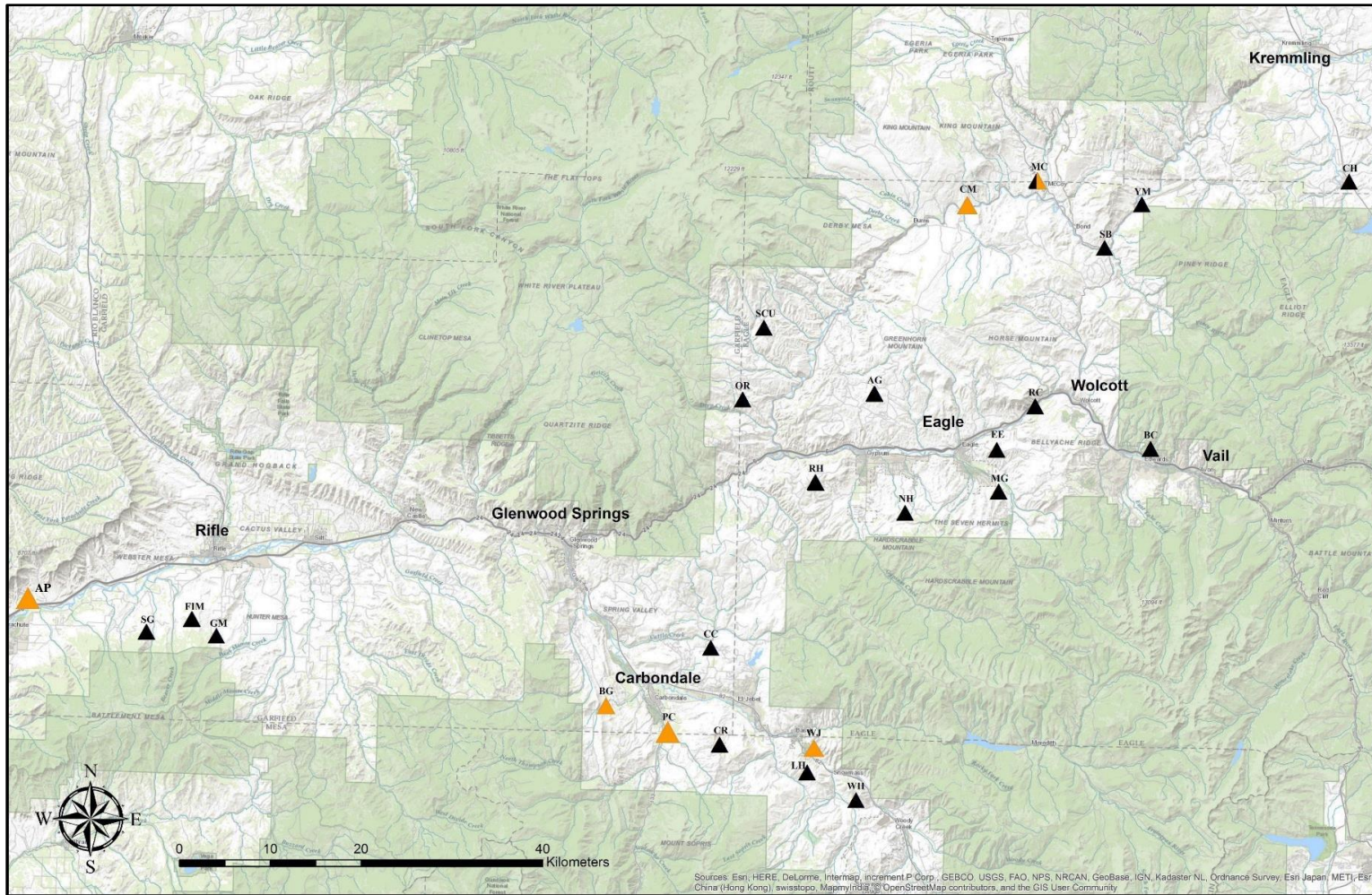
Figure 3. Map for 26 collection site for *P. harringtonii* and *P. osterhoutii*.
Collections were completed during June in 2015 and 2016 where triangles represent locations of sampling collections.
Black triangles represents *P. harringtonii* populations and orange represents *P. osterhoutii* populations.

Microsatellite Analysis

Extractions. Deoxyribonucleic acid (DNA) was successfully extracted using a modified cetyltrimethylammonium bromide (CTAB) method that uses the addition of Caylase to break down secondary compounds (Doyle 1987; Friar 2005).

Polymerase Chain Reactions (PCR). Seventeen microsatellite loci were selected from Kramer et al. (2007; 2011) and Dockter et al. (2013), and a universal tag (M13, CAGT, or T7term) was added to the 5' end of one of the flanking primers to allow for fluorescent labeling of products following the procedure of Boutin-Ganache et al. (2001). Primer pairs were optimized for annealing temperature and $MgCl_2$ concentration. Six primers optimized from Kramer and Fant (2007), one primer from Kramer et al. (2011) and two primers from Dockter et al. (2013) were determined to be variable for *Penstemon harringtonii* (Table 2).

Table 2. Primer characteristics and reaction conditions for nine variable microsatellite markers for *Penstemon harringtonii*. Source corresponds to Kramer and Fant (2007), Kramer et al. (2011) and Dockter et al. (2013) where original primer sequences were published.

Primer	Repeat	Tag	DNA	$MgCl_2$	Anneal Temp. (ºC)	Source
PS005	$(GAA)_6$	M13	1 µl	2 µl	52.9	Dockter et al. (2013)
PEN06	$(TG)_9(GA)_{12}$	T7term	1 µl	3 µl	50.9	Kramer et al. (2011)
PS034	$(AC)_9$	CAGT	1 µl	1.5 µl	50.9	Dockter et al. (2013)
PEN23	$(GA)_{21}$	T7term	1 µl	2.5 µl	55.1	Kramer and Fant (2007)
PEN02	$(TC)_{14}(CA)_{13}$	CAGT	1 µl	2 µl	52.9	Kramer and Fant (2007)
PEN04	$(TC)_{22}$	T7term	1 µl	1 µl	55.1	Kramer and Fant (2007)
PEN05	$(TC)_{25}$	CAGT	0.5 µl	2 µl	52.9	Kramer and Fant (2007)
PEN18	$(CT)_{20}(CA)_{20}$	T7term	1 µl	1.75 µl	52.9	Kramer and Fant (2007)

Amplification of microsatellite loci was performed in 12 µL reaction volumes containing: 2.4µL 5X GoFlexi buffer (Promega, Madison, Wisconsin), 0.7 µL dNTP mixture (2.5 mM, Promega), 0.6 µL non-tagged primer, 0.6 µL tagged primer, 0.6 µL

fluorescent tag, 1-3 µL Magnesium chloride ($MgCl_2$), 0.06 µL BSA (Bovine Serum

Albumin, 100X, Promega), 0.06 µL GoFlexi Taq polymerase (Promega), 0.5-1 µL of

genomic DNA, and 3.48 - 4.98 µL dH_2O (Table 2). For all primers, PCR amplification

was carried out on a Mastercycler proS thermal cycler (Eppendorf, Hamburg, Germany).

An initial denaturation at 94ºC for 1 minute followed by 35 cycles of denaturation at

94ºC for 1 minute, annealing at primer-specific temperature for 1 min and a primer

extension at 72ºC for 1 minutes, with a final extension step of 30 minutes at 72ºC.

Products were verified via electrophoresis using a 1% agarose gel and then multiplexed

where possible and analyzed on a 3730XL Genetic Analyzer (Applied Biosystems, Foster

City, CA) at Arizona State University. Products were loaded along with GeneScan

500LIZ Size Standard (Applied Biosystems) according to manufacturer's specifications.

Fragment peak scoring for all primers was completed using Geneious 8.0.3 (Biomatters

Limited, Auckland, New Zealand).

Statistical Analysis

GENALEX 6.5 (Peakall and Smouse 2006; Peakall and Smouse 2012) was used

to calculate average number of alleles (N_a), effective number of alleles (N_e), observed

heterozygosity (H_o), expected heterozygosity (H_e), inbreeding coefficient (F_{IS}), pairwise

number of migrants shared between populations (N_m) and pairwise genetic distance

between populations (F_{ST}). Principle component analysis (PCoA) was generated as well.

Population structure was determined by using the Bayesian cluster analysis

program STRUCTURE 2.3.4 (Pritchard et al. 2000). Burn-in and run lengths of 100,000

replicates were used for each STRUCTURE analysis. Values for K=1 to K=10 for 15

replicates were inputted into STRUCTURE HARVESTER which determined the inferred

number of populations (K) (Earl and vonHoldt 2012). The Evanno et al. (2005) method

of determining ideal K is implemented by STRUCTURE HARVESTER, which

determines the appropriate K by the second order rate of change with respect to K of the

likelihood function. The greatest delta K value is an indication of the best-supported K

value (Earl and vonHoldt 2012). STRUCTURE analysis was completed for populations

of *P. harringtonii* only as well as a combination of *P. harringtonii* and *P. osterhoutii*

populations. GENELAND (Guillot et al. 2005) was used to analyze geographic

coordinate data and multilocus genotypes to determine genetic discontinuities between

populations across the landscape. The analysis was run for 1×10^6 iterations, samples

were thinned every 1000 iterations and a post-process burn-in of 250 was used.

EDENetwork: Ecological and Evolutionary Networks (Kivelä et al. 2015) utilizes the

population genetic metric F_{ST} to construct a distance/dissimilarity matrix, which was used

to build a minimum spanning tree among sampled populations. POPTREEW (Takezaki et

al. 2014) was used to construct a neighbor-joining tree using Nei's D_A (Nei et al. 1983)

with 1000 bootstrap replicates. The phylogenetic tree was exported and edited using

Figtree v1.4 (Rambaut 2012).

Results

DNA extractions were successful for 734 individuals. All nine microsatellite loci

were viable and polymorphic among populations.

Diversity

Calculations for average number of alleles (Na), effective number of alleles (Ne),

observed heterozygosity (Ho), expected heterozygosity (He) and inbreeding coefficient

(FIS) for each population of *P. harringtonii* are shown in Table 3. The average number

of alleles and effective number of alleles across all populations of *P. harringtonii* was 9.056 and 5.216, respectively. Mayer Gulch had the highest average number of alleles (11.778) and effective number of alleles (7.455). Cattle Creek Rd had the lowest average number of alleles (4.000) and effective number of alleles (2.822). The average observed and expected heterozygosity across all population of *P. harringtonii* populations was 0.587 and 0.706, respectively. East Eagle had the highest observed heterozygosity (0.667) and Grass Mesa had the lowest observed heterozygosity (0.494). Mayers Gulch had the highest expected heterozygosity (0.761) and Cattle Creek Rd had the lowest (0.582). The average inbreeding coefficient (F_{IS}) across all *Penstemon harringtonii* populations was 0.154. The lowest F_{IS} was in Cattle Creek Rd (0.031) and the highest F_{IS} was in Grass Mesa (0.280).

Pairwise genetic distance (F_{ST}) was calculated between all pairs of regional groups (Table 4). The average F_{ST} between regions was 0.0508, with the highest values between Rifle and RFRV (0.081) and the lowest between Eagle and NCORV (0.033). The average F_{ST} within regions was 0.0372, the highest values within RFRV (0.066) and the lowest within Eagle (0.019). Number of migrants (N_m) was calculated between for all pairs of regional groups (Table 4). The average N_m was 4.713, with the N_m among regions between Eagle and NCORV (8.125) and the lowest between Rifle and RFRV (3.301). The average Nm within regions was 8.615, the highest values within Eagle (13.686) and the lowest within RFRV (4.573).

Table 3. Genetic diversity statistics from all sampled populations of *P. harringtonii* for nine microsatellite loci.

Population	Region	N	N_a	N_e	H_o	H_e	F_{IS}
P. harringtonii[1]							
Agnew Gulch	Eagle	32	10.889	6.364	0.613	0.713	0.114
Berry Creek	Eagle	32	9.333	5.015	0.571	0.697	0.159
East Eagle	Eagle	32	11.556	6.286	0.667	0.753	0.096
Sheep Creek Uplands	Eagle	32	11.222	6.526	0.594	0.757	0.201
Mayer Gulch	Eagle	32	11.778	7.455	0.603	0.761	0.194
North Hardscrabble	Eagle	32	10.556	5.913	0.602	0.733	0.159
Onion Ridge	Eagle	32	10.222	6.171	0.572	0.739	0.194
Red Canyon	Eagle	32	10.333	6.283	0.523	0.728	0.239
Red Hill	Eagle	32	10.333	5.876	0.574	0.728	0.186
McCoy	NCORV	12	6.889	4.886	0.597	0.718	0.173
CO10H9	NCORV	33	8.111	4.831	0.572	0.742	0.217
State Bridge	NCORV	32	9.889	4.699	0.588	0.697	0.135
Yarmony	NCORV	32	10.444	6.124	0.572	0.732	0.196
Light Hill	RFRV	32	7.333	4.264	0.642	0.698	0.056
Crown	RFRV	32	8.333	4.601	0.602	0.714	0.158
Williams Hill	RFRV	32	5.222	2.921	0.532	0.585	0.126
Cattle Creek Rd	RFRV	6	4.000	2.822	0.574	0.582	0.031
Spruce Gulch	Rifle	32	8.444	4.671	0.604	0.676	0.094
Grass Mesa	Rifle	32	7.889	4.232	0.494	0.692	0.280
Flat Iron Mesa	Rifle	32	8.333	4.382	0.637	0.683	0.066
Mean		**29**	**9.056**	**5.216**	**0.587**	**0.706**	**0.154**

[1] highlighted values are representative of ideal values for conservation purposes

Table 4. Relative measurement of genetic distance (F_{ST}) above the diagonal and number of migrants (N_m) below the diagonal, between regional groups.

N_m / F_{ST}	Eagle	NOCRV	E_of_GlenCYN[1]	RFRV	Rifle
Eagle	*13.686/0.019*	0.033	**X**	0.061	0.047
NOCRV	*8.125*	*7.039/0.037*	**X**	0.074	0.063
E_of_GlenCYN[1]	**X**	**X**	*10.608/0.027*	0.065	0.052
RFRV	*4.205*	*3.372*	*3.949*	*4.573/0.066*	0.081
Rifle	*5.371*	*3.894*	*4.916*	*3.301*	*9.162/0.027*

[1] **X** indication of no data because E_of_GlenCYN is a combination of Eagle and NOCRV so comparisons weren't made with those regions.

Genetic Structure

Bayesian cluster analysis using STRUCTURE was run for all individuals, which

included all *P. harringtonii* and *P. osterhoutii* samples, and for only *P. harringtonii*.

STRUCTURE HARVESTER determined that K=2 or K=3 are the most probable assignment for the data set including *P. harringtonii* and *P. osterhoutii* individuals (Figure 4). The STRUCTURE analysis of the *P. harringtonii* and *P. osterhoutii* populations divided the data into two distinct clusters (Figure 5A) clearly distinguishing all *P. harringtonii* populations (yellow) and *P. osterhoutii* (blue), or three distinct clusters (Figure 5B), which further separated the *P. harringtonii* populations into east (brown) and west (purple) of Glenwood Canyon and kept *P. osterhoutii* (blue) as a distinct group. McCoy (MC), Catamount (CM), Wingo Junction (WJ) and Barber's Gulch (BG) were all collected as *P. harringtonii* but ended up being all or partially *P. osterhoutii* according to genetic analysis. McCoy (MC) is represented in both *P. harringtonii* and *P. osterhoutii*, and even though these samples were collected where individuals were inhabiting the same area significant admixture is not observed.
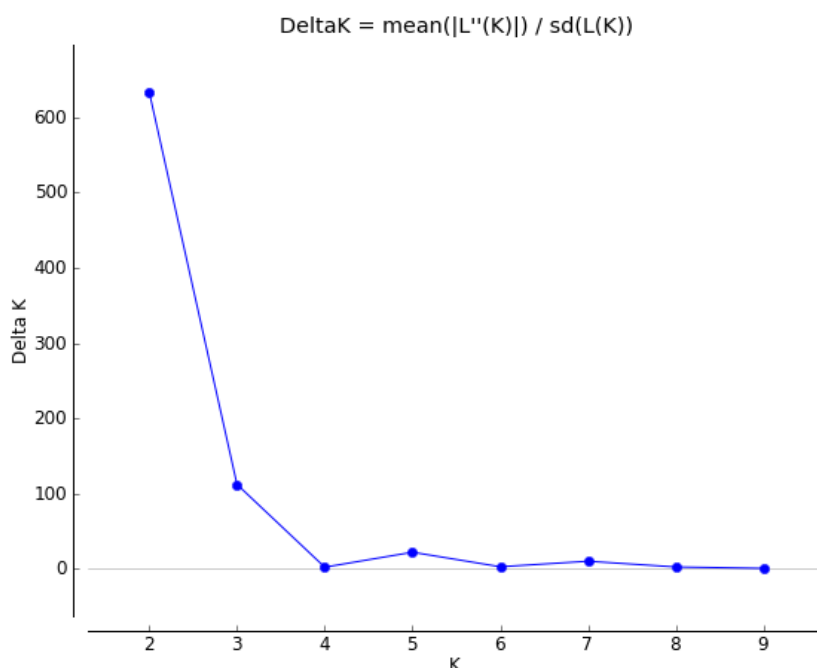
Figure 4. STRUCTURE HARVESTER for *P. harringtonii* and *P. osterhoutii* combined data set.
The graph is indicating the rate of change in likelihood calculated using the Evanno et al. (2005) method for each K value assigned.
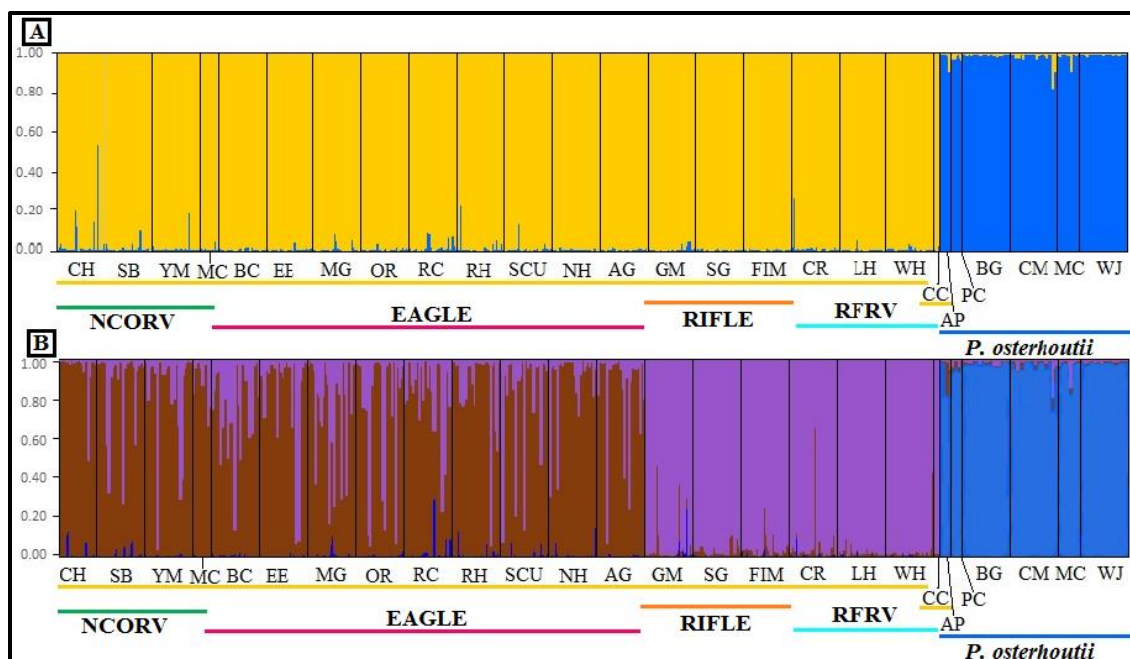
Figure 5. Bar plot images of the STRUCTURE results for *P. harringtonii* and *P. osterhoutii* combined data set.
The two graphs represent two different K-values: A) K=2, blue=*P. osterhoutii* individuals and yellow = *P. harringtonii* individuals B) K=3, blue=*P. osterhoutii*, purple=*P. harringtonii* west of the Glenwood Canyon and brown=*P. harringtonii* east of Glenwood Canyon

For *P. harringtonii* only dataset STRUCTURE HARVESTER had maximum

support for K=2 and K=3, with K=4 also showing an elevated rate of change (Figure 6).

The best-supported STRUCTURE pattern was K=2 (Figure 7A) which divided the

populations into east of Glenwood Canyon (E_of_GlenCYN; brown) and west of

Glenwood Canyon (W_of_GlenCYN; purple). STRUCTURE analysis for K=3 (Figure

7B) was also highly supported, displaying populations east of Glenwood Canyon

(E_of_GlenCYN; brown) together and splitting Rifle (orange) and RFRV (teal)

populations. Additionally, K=4 (Figure 7C) is included too as it showed elevated

support, which shows that same pattern as K=3, but with the east of Glenwood section

split between NCORV (green) and Eagle (pink) populations as designated earlier in Table

3. The east of Glenwood Canyon split is supported, but substantial admixture is present

between the Eagle and NCORV regions. Admixture to some degree can be seen in each region, but in NCORV (green) the CO10H9 (CH) and in RFRV (teal) the Williams Hill (WH) populations are distinctly void of admixture.
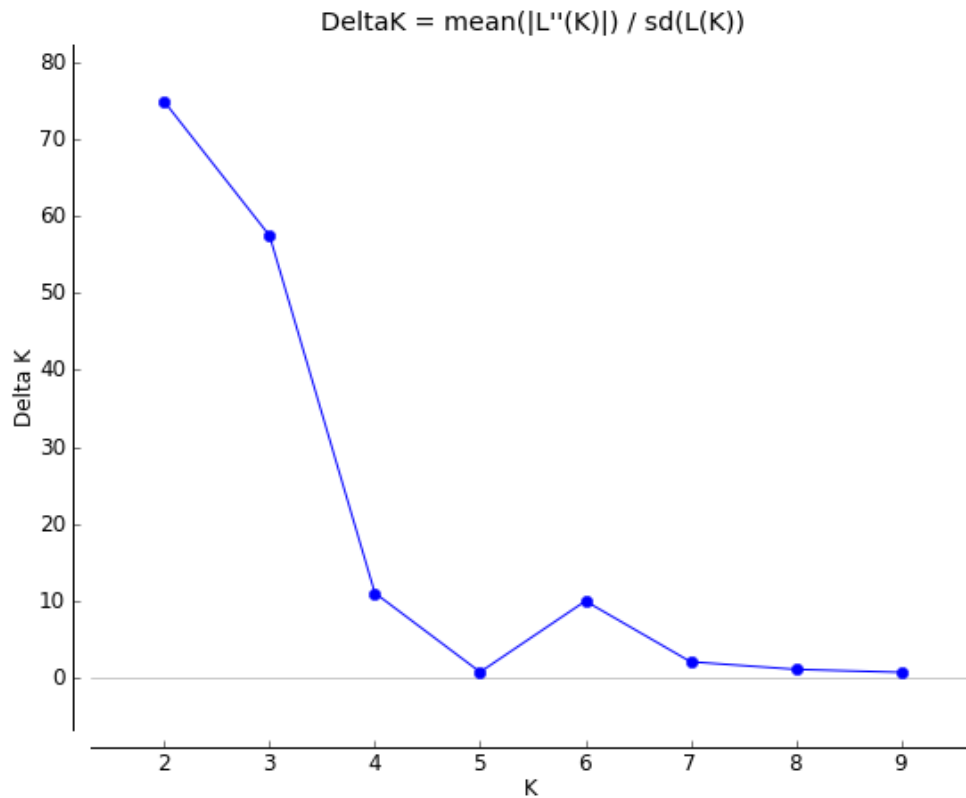
Figure 6. STRUCTURE HARVESTER for *P. harringtonii* only data set.
The graph is indicating the rate of change in likelihood calculated using the Evanno et al. (2005) method for each K value assigned.
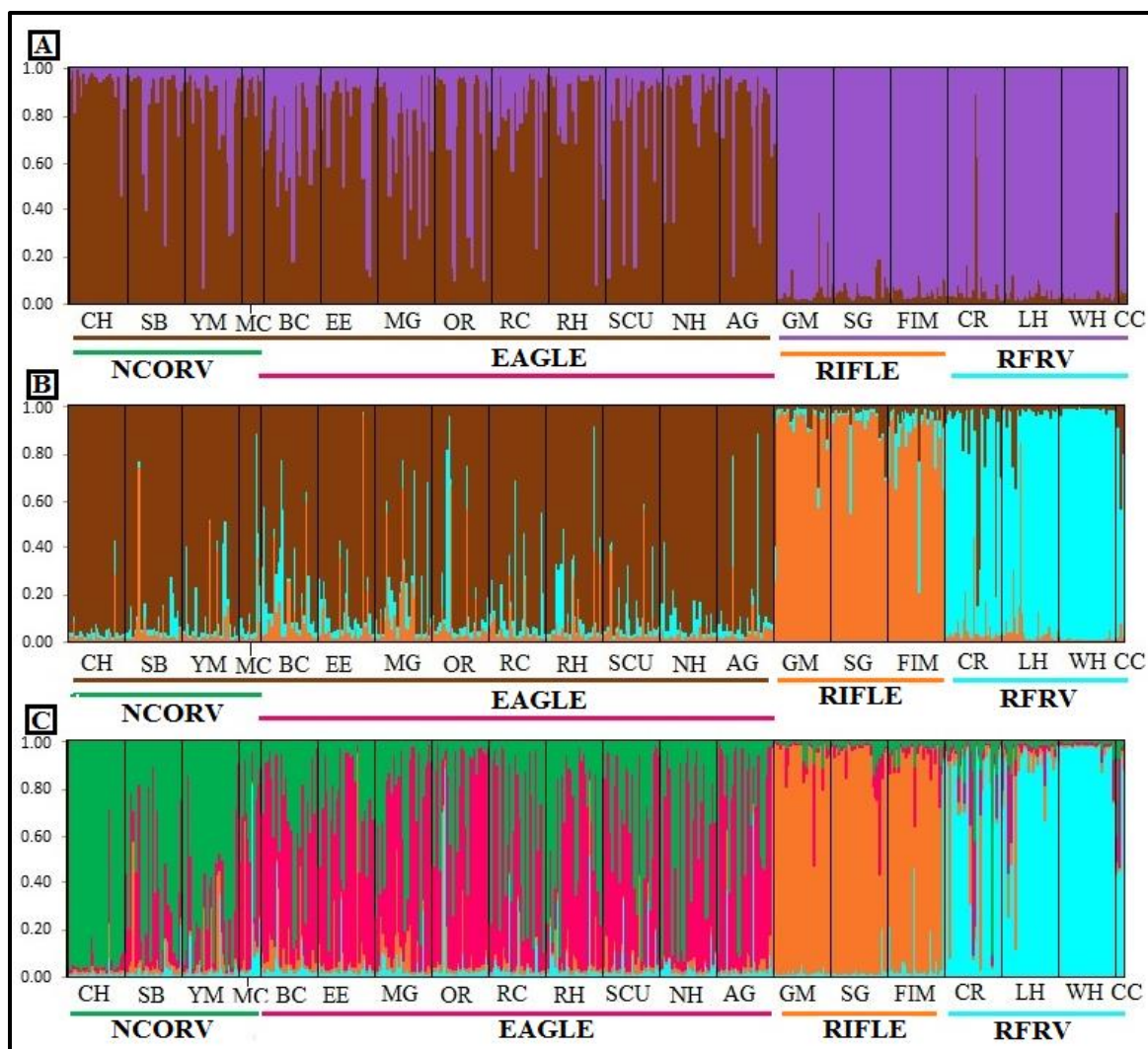
Figure 7. Bar plot images of the STRUCTURE results for *P. harringtonii* only.
The three graphs represent three different K-values: A) K=2; brown=E_of_GlenCYN,
purple=W_of_GlenCYN, B) K=3; brown=E_of_GlenCYN, orange=Rifle, teal=RFRV,
C) K=4; green=NCORV, pink=Eagle, orange=Rifle, teal=RFRV

The Principle Coordinate Analysis (PCoA) for the 20 populations of *P.*

harringtonii resolves three clusters (Figure 8), which corresponds to the second highest

supported STRUCTURE diagram (Figure 7B). The variation represented by Coord. 1

(x), Coord. 2 (y), and Coord. 3 (z) (not shown) are 26.98 %, 24.62% and 13.22%,

respectively. The Rifle populations are colored orange and outlined in an orange circle,

the RFRV populations are colored in teal and outlined in a teal circle, and Eagle (pink)

and NCORV (green) populations all grouped together and are outlined in a brown circle.

The grouping of Eagle and NCORV in the PCoA, provides high support for the K=3

STRUCTURE analysis (Figure 7B) where Eagle and NOCRV grouped together, and the

high levels of admixture throughout the two regions in the K=4 STRUCTURE analysis

(Figure 7C) also supports the notion that Eagle and NCORV are acting as one population.

Williams Hill (WH) is isolated from the other members of the RFRV groups which

corresponds to a K=9 STRUCTURE analysis (not shown) in which WH is a unique

group separated from the rest of RFRV populations.
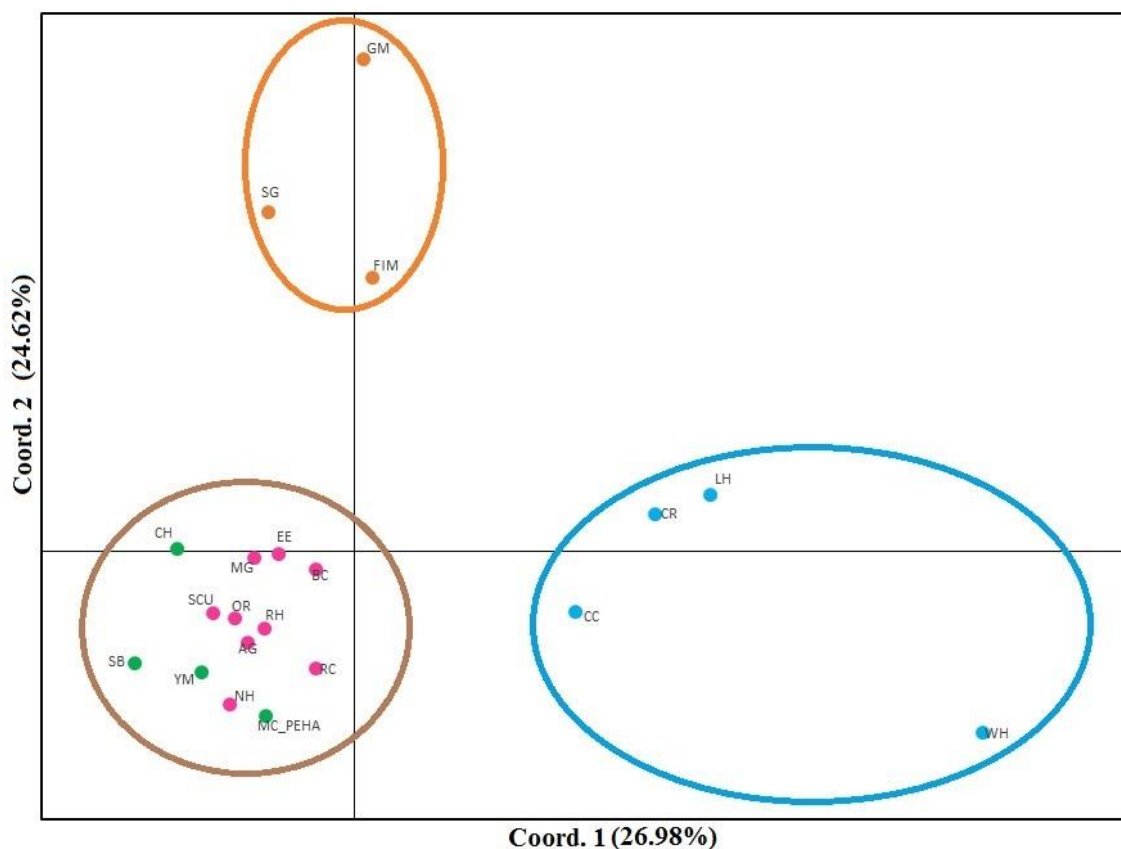
Figure 8. Principle Coordinate Analysis (PCoA) of 20 populations of *P. harringtonii.*
The first two coordinates are plotted with variation shown on Coord. 1 (x) and Coord. 2
(y) and Coord. 3 (z) (not shown) as 26.98%, 24.62% and 13.22 % respectively

GENELAND (Guillot et al. 2005) was used to determine genetic relationships

based on individual geographic coordinates with microsatellite genotypes, grouping

sampled populations to locate genetic discontinuities between populations throughout the

sampling area. The software determined that six genetic/geographic clusters was the

most meaningful grouping. Figure 9 gives a summary of the posterior probabilities and

displays population membership for each of the six regions. Table 5 displays pairwise

F_{ST} values among the 6 genetic/geographic clusters. These values ranged from 0.0078

between Eagle regions NCORV regions, cluster 2 and cluster 3 (SB population), to

0.0604 cluster 5 (CH population) and cluster 6 (WH population). A series of maps

(Figure 10) display the posterior probability of each of the 6 clusters with the most

similar assignments indicated by bright white, moderately similar in yellow and least

similar in red. The three populations near Rifle (GM, FIM and SG) make up cluster 1.

The CH, SB and WH populations were all identified as unique, clusters 5, 3 and 6,

respectively. All of the populations in RFRV except WH are identified in cluster 4. The

Eagle and NCORV continuity were partial reaffirmed in cluster 2 which included all of

Eagle populations plus MC and YM populations of NCORV.
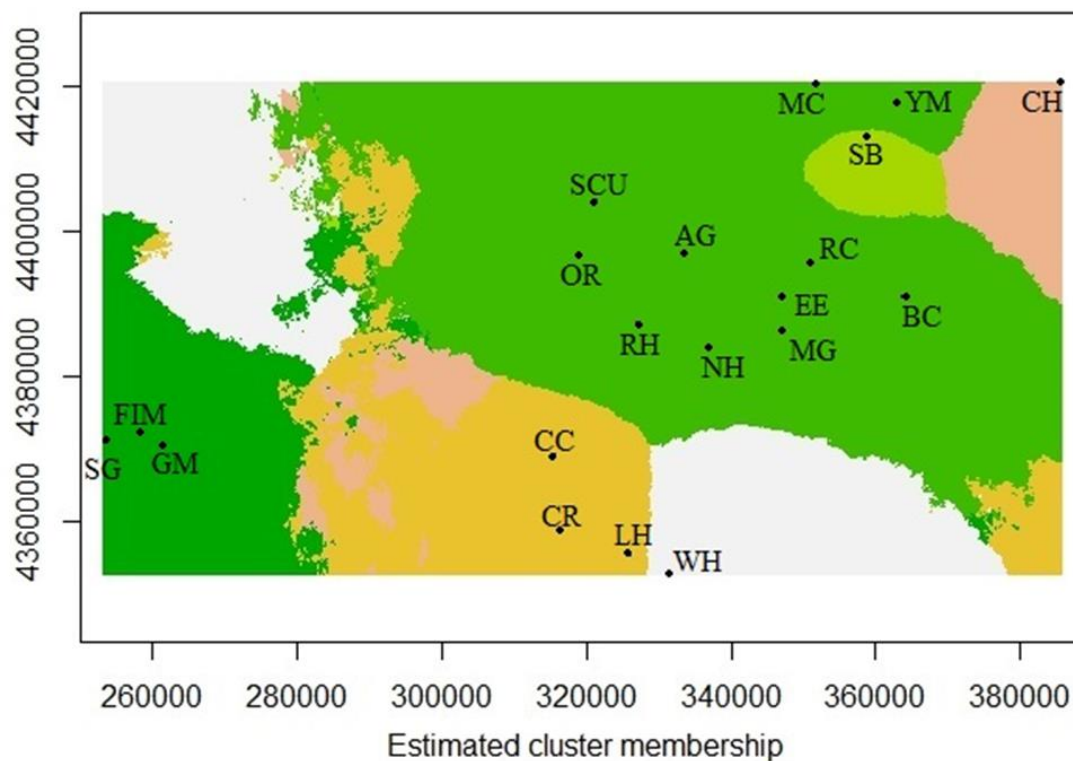
Figure 9. Map of *P. harringtonii* population's membership according to posterior probability.
Colors coordinate with the six GENELAND clusters outlined in Figure 10. Cluster 1, dark green (FIM, SG, GM); Cluster 2, green (BC, RC, EE, MG, NH, RH, OR, AG, SCU, MC, YM); Cluster 3, lime green (SB); Cluster 4, tan (CR, LH, CC); Cluster 5, light pink (CH); Cluster 6, white (WH).

Table 5. Relative measurement of genetic distance (F_{ST}) between GENELAND clusters identified in Figure 10.

F_{ST}	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
Cluster 1	0	0.0152	0.0259	0.0252	0.0254	0.0575
Cluster 2		0	0.0078	0.0180	0.0158	0.0405
Cluster 3			0	0.0329	0.0188	0.0561
Cluster 4				0	0.0287	0.0360
Cluster 5					0	0.0604
Cluster 6						0
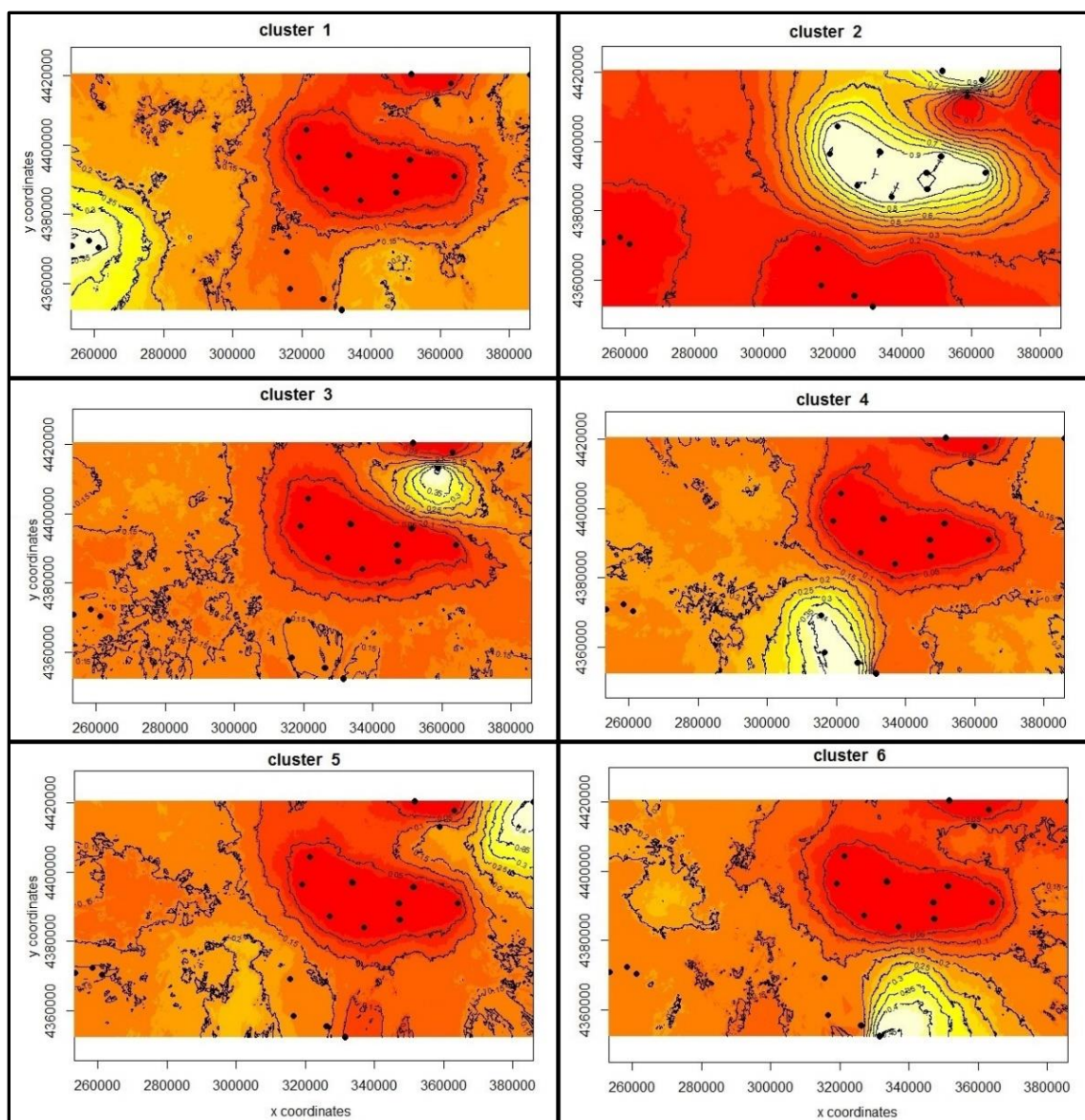
Figure 10. GENELAND posterior probability assignment of genetic discontinuities of the *P. harringtonii* populations.
High levels of genetic similarity is indicated by bright white, moderate levels by yellow and low levels of similarity by red. Scale on the shown axes is geographic coordinates.

A minimum spanning tree for all 20 populations of *P. harringtonii* was generated

by EDENetwork. The tree (Figure 11) is fully connected and undirected, giving a look at

how each population is connected, and which population may be essential for the

continuation of the gene flow in specific areas of the range of *P. harringtonii*. Line

thickness is an indication of the number of possible connection that have been

consolidated, the line shown being the most ideal relationship with the surrounding

nodes. Agnew Gulch (AG) is a central population from which the majority of the Eagle

populations radiate from, as well as the NCORV population, reaffirming the continuity

between the two regions and further supporting STRUCTURE and GENELAND

analysis. The Sheep Creek Uplands population is acting as a primary connection point

for the bulk of Eagle gene pool to the remainder of NCORV populations as well as

connecting to the rest of the Eagle populations. Mayers Gulch is the final connection for

gene flow to make it throughout the rest of the range of the species, connecting to the

RFRV and Rifle populations.

The POPTREEW phenogram (Figure 12) displays distinct separation of the Rifle,

RFRV and all population east of Glenwood Canyon further supporting the combination

of the Eagle and NOCRV regions. The branches that represent Eagle and NCORV

populations have very low bootstrap support, indicating not enough distinctiveness is

available to support the given relationship with this analysis. The Rifle and RFRV

separation is supported fairly well with a bootstrap value of near 80%. Additionally,

Williams Hill (WH) and CO10H9 (CH) both have long branches showing distinctiveness

within each of their clades, further supporting GENELAND cluster analysis.
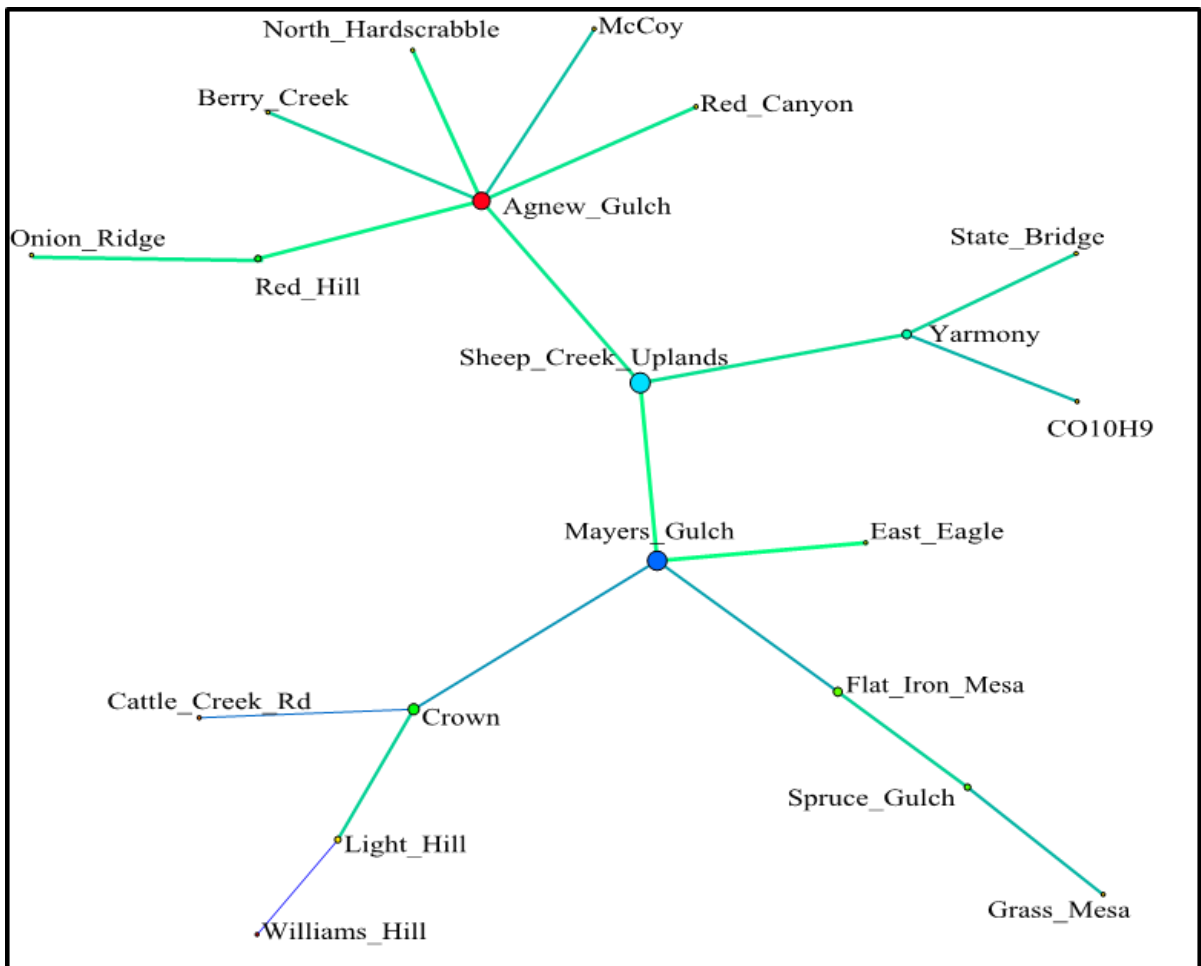
Figure 11. Minimum spanning tree derived from genotype matrices.
The tree is based allele frequency data using F_{ST} distance measured using EDENetwork software. The size of the node indicates the relative amount of gene flow occurring through the node and the thickness of the line is an indication of the amount gene flow between the two nodes it connects
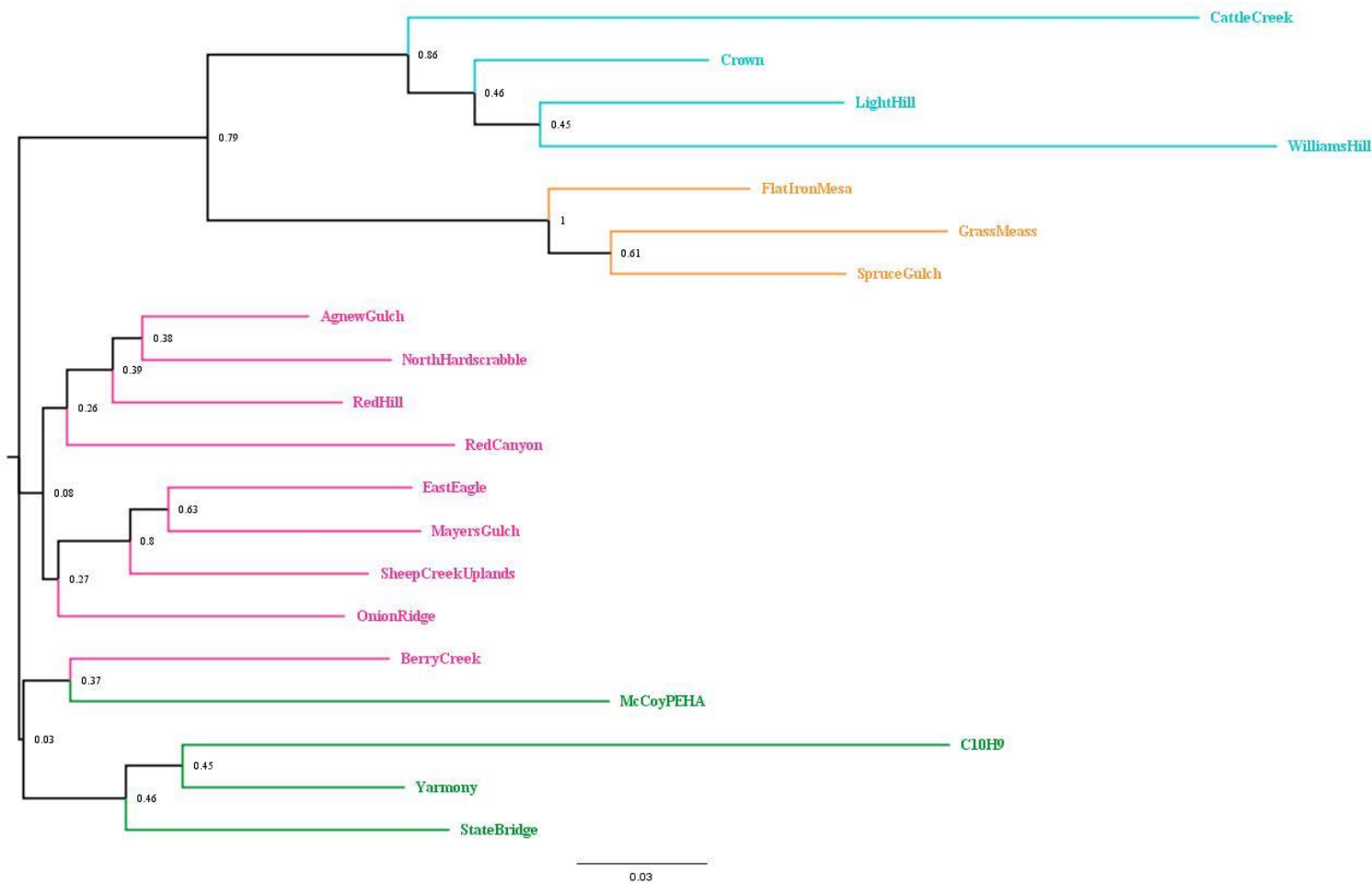
Figure 12. Phenogram of *P. harringtonii* populations.
Constructed in POPTREEW utilizing Nei's D_A genetic distance to determine genetic distinctiveness to aid the neighbor-joining methods in constructing the tree based on allelic frequency data from nine microsatellite loci.

Discussion

Endemic species are often rare by definition, with a restricted range and small population sizes, resulting in low genetic diversity and limited ability to respond to stochastic events or persistent disturbance (Schemske et al. 1994). Anthropogenic activities may result in habitat alterations which may create more or less gene flow between regions. This manipulation of gene flow patterns is a departure from the natural process therefore altering the evolutionary path of the organism by stopping or solidifying differentiation between populations of the species of concern (Crandall et al. 2000; Fraser and Bernatchez 2001; Aguilar et al. 2008). *Penstemon harringtonii* is under increased pressure from anthropogenic activities due to the compounding effects of grazing, oil and gas development, and increasing recreation activities as urban development continues, leading to habitat reductions throughout its range (Panjabi and Anderson 2006; Elliott et al. 2009; Neely et al. 2009). Land management action plans are needed for this species, that provide options to maintain genetic variablity within and among populations through the consideration of connectivity and diversity within and among known populations.

Genetic Structure

Group assignments for the 20 populations of *Penstemon harringtonii* were defined through the use of STRUCTURE, GENELAND, PCoA, and phenogram. The STRUCTURE analyses showed the greatest support for two genetic groups, dividing populations east and west of Glenwood Canyon (Figure 7A), but also provided relative high levels of support for three genetic groups, further separating Rifle and RFRV (Roaring Fork River Vaelly) populations west of Glenwood Canyon (Figure 7B).

Principal Component Analysis also supported three genetic groups (Figure 8), as did the phenogram (Figure 12), but with limited bootstrap support. The RFRV genetic group that was determined from the PCoA incorporates the WH population even though it is separated from the others suggesting some differentiation, which can be seen in the GENELAND analysis (Figure 9 and 10). GENELAND analysis partially supported the consolidation of Eagle and NCORV (Northern Colorado River) in cluster 2 where all Eagle populations and two NCORV populations grouped together and the pairwise F_{ST} with third NCORV population (Cluster 3) was the lowest of all the pairwise comparisons (Table 5, Figure 10; 0.0078). These five analyses effectively support or partially support the notion that the best representation of the 20 populations of *P. harringtonii* is three groups: Rifle, RFRV and E_of_GlenCYN.

Genetic divergence among population can effectively be measured using Wright's F-Statistic (F_{ST}) (Holsinger and Weir 2009). According to Freeland et al. (2011) values of 0.0 – 0.05 indicate little genetic differentiation, 0.05 – 0.25 moderate genetic differentiation and over 0.25 indicate marked genetic differentiation. Hey and Pinho (2012) looked at populations of insects, birds, mammals and plant to define how divergence was determined and concluded a threshold F_{ST} value of 0.35, above were species, below subpopulations. Plant genomes often allow alleles to be transferred across species via hybridization which may dilute signal between two closely related species, resulting in misinterpretation of genetic differentiation measurements. Therefore various measurements, including genetic drift, gene flow and number of migrants, must all be considered in addition to F_{ST} to conclude if genetic differentiation is present (Muir et al. 2012). F_{ST} thresholds differ throughout the primary literature depending on the species of

interest and the type of organism. Following the threshold put forth by Hey and Pinho (2012) and the guidelines of Freeland et al. (2011) all of the pairwise F_{ST} values among regions of *P. harringtonii* weren't near any of the thresholds for species distinction, indicating all populations are *P. harringtonii* form a cohesive species. The pairwise F_{ST} values indicate little differentiation between all populations sampled, but the three major regional groups show a slight elevation in genetic differentiation, which is seen to validate the proposed population structure. Two populations (cluster 5 and 6) recognized in GENELAND (Figure 10) may indicate localized distinction, but nothing on the level of speciation. Cluster 5 (CH) had pairwise F_{ST} values on par with other region group's values, but cluster 6 (WH) pairwise F_{ST} values ranged from 0.036 – 0.0604, which was greater than any of the other pairwise comparison values indicating early development of genetic differentiation.

Number of migrants is the number of breeding adults that are moving between populations or regional groups (Freeland et al. 2011). Small populations are at risk of reduced genetic diversity (Schemske et al. 1994) unless gene flow recharges the gene pool of the population with migrants (Vucetich and Waite 2000). To offset the extinction risk and loss of genetic diversity, a certain number of migrants are necessary. The rule used is that one immigrant per generation will introduce sufficient new genetic material to prevent divergence among populations (Vucetich and Waite 2000; Wang 2004). The number of migrants (N_m) between regional groups range from 3.301 – 4.916 migrants per generation, therefore *P. harringtonii* is well over the standard of one migrant per generation. The minimum spanning tree (Figure 11) display's prevalent gene flow among the populations of the Eagle region with one of the NCORV populations clumping

with the Eagle region populations but still maintaining some structure of the NCORV groups. The genetic distances and gene flow between populations and regional groups of *P. harringtonii* indicate that is a single species, but structure does exist and slight differentiation is present.

Diversity

Rare and endemic species are of concern to land management agencies as a reduction in population size can lead to reduced genetic diversity, resulting in limited resilience within populations or the species as a whole, depending on the scale of disturbance (Schemske et al. 1994; Freeland et al. 2011; Sterling et al. 2012; Zarlenga et al. 2014). Populations that were sampled were perceived to be small and isolated when in fact, they might be substantially larger and have landscape level connections that allow for a respectable level of genetic diversity. A heterozygosity of 1 indicates no shared alleles and high genetic diversity, while a 0 indicates no variability at all. Generally, heterozygosity of 0.3 is indicative of moderately high genetic diversity (Nybom 2004). All populations of *P. harringtonii* have heterozygosity above 0.3, ranging from 0.494 – 0.667 for observed heterozygosity (H_o) and 0.582 – 0.761 for expected heterozygosity (H_e) (Table 3). The data for all populations of *P. harringtonii* indicate high genetic diversity.

Inbreeding results in the accumulation of mildly deleterious alleles by increasing the frequency that they are found in a homozygous state (Freeland et al. 2011). These mildly deleterious alleles will not be purged from populations effectively due to a lack of strong selective pressure, but persist in the population (Freeland et al. 2011). Inbreeding coefficients (F_{IS}) of 0.50 or lower is considered to be of little concern among plant

populations due to the ability of many plants to self-fertilize, while values greater than 0.50 can lead to inbreeding depression resulting in a loss of genetic diversity. The inbreeding analysis for *P. harringtonii* indicates very little inbreeding is occurring within populations (Table 3). The highest inbreeding is observed at Red Canyon (0.239), CO10H9 (0.217), and Grass Mesa (0.280), which all can be argued to be isolated populations that may have reduced gene flow. Red Canyon is flanked by I-70 and the Eagle River, with HWY 6 splitting it up the middle, which results in a scenario of heavy disturbance from foot traffic or invasive species pressure due to close proximity to corridors to transport those directly to the site with minimal effort. The CO10H9 site is the furthest north site with the closest other sampled site being Yarmony about 10 miles away. The Grass Mesa site is isolated due to habitat removal and extensive oil and gas development resulting in high inbreeding rates. Overall, inbreeding levels within *P. harringtonii* populations are low with a few populations having slightly elevated value, but nothing that would result in inbreeding depression.

Other *Penstemon* Species

Penstemon harringtonii has a limited range, but within that range several other species of *Penstemon* species are present. Most of these species are easily distinguishable from *P. harringtonii* in all life stages, but *P. osterhoutii* is the exception; it is difficult to distinguish from *P. harringtonii* if flowers are absent. Due to this, during field collections, four populations (MC, CM, WJ and BG) were collected that were genetically identified *P. osterhoutii* or a combination of *P. harringtonii* and *P. osterhoutii*. Through genetic analyses, all populations were delineated correctly with the McCoy (MC) population being a combination of the two species with limited genetic

admixture (Figure 5A) indicating that these two species are good species that rarely hybridize if at all.

Microsatellite data has been used to investigate relationships between *Penstemon* species with various pollination syndromes (Kramer et al. 2011), to determine vulnerabilities in rare *Penstemon* species (Wolfe et al. 2014; 2016) or to confirm taxonomic relationships between morphologically similar *Penstemon* species (Johnson et al. 2016). Wolfe et al. looked at *P. debilis* (2014) and *P. albomarginatus* (2016) to assess the genetic structure and diversity of these two rare and endemic species to inform conservation decisions. Analysis from both taxa resolved geographic structure among populations, with admixture seen between the Nevada and Arizona populations of *P. albomarginatus,* similar to the admixture between regional groups of *P. harringtonii*. In contrast, *P. debilis* exhibited minimal admixture. These two studies provide flanking examples to this study with *P. debilis* having smaller population and a limited range within a single county, and *P. albomarginatus* has a larger range spanning across three states. Kramer et al. (2011) looked at three common species of *Penstemon* to determine the effects of pollination syndrome and landscape on genetic structure. Kramer et al. (2011) looked at three *Penstemon* species with three different flower morphologies, which attracted small bees, big bodied bees and hummingbirds, which each represented different vector for pollen distribution across the landscape. Kramer et al. (2011) showed that landscape is an important determinant of genetic structure and the type of pollinator can determine the level of genetic structure for a species. The bigger pollinators traveled greater distances, resulting in greater admixture between populations and limited genetic structure. Small pollinators traveled shorter distances, therefore genetic material was not

admixed among populations as often, resulting in more defined genetic structure

correlating to landscape barriers. Landscape level geography determined the major

structure of *P. harringtonii* just as it did in Kramer et al. (2011) analyses. The relatively

clear delineation between the three regions of *P. harringtonii* (Figure 7B) may be a result

of dependence on medium sized pollinators (Panjabi and Anderson 2006) that can

overcome distances within regions, but can't effectively reduce the structure between

regions due to geographic features. Neilson (1998) determined that *P. harringtonii* has

pollinator redundancy built in as each year different pollinators were seen to be the

dominate visitors to the flowers. Neilson (1998) did conclude that medium sized bees

(Megachilidae family) and wasps (Vespidae family, Masarinae subfamily) were the main

pollinators. Genetic data indicates that other pollination vectors may be contributing to

long distance gene flow between regions. The variability in pollinators that utilize *P.*

harringtonii allows for gene flow and genetic diversity to remain high within and

between regions. Finally, Johnson et al. (2016) determined taxonomic relationships

between morphologically similar *Penstemon* species by sampling and determining

genetic structure via STRUCTURE and PCoA. Similar analyses were done for *P.*

harringtonii to determine regional structure and to confirm misidentified populations as

P. osterhoutii.

Conservation and Management

Penstemon harringtonii is a rare and endemic species that may be more abundant

and more diverse than first perceived. To ensure that anthropogenic actions and activities

don't reduce this species population numbers, appropriate conservation and management

actions should be implemented. The three regional groups of *P. harringtonii* should be

the main focus of any conservation actions, as this provides a broad representation of the

species across its range. Within each of these regional groups, E_of_GlenCYN, RFRV,

and Rifle, a subset of populations should be identified that represent high levels of

genetic diversity and low levels of inbreeding to ensure that the most resilient group of

individuals are selected for conservation. Recommendations for which of the populations

utilized for this study should be targeted for conservation follow.

The Rifle region only includes three populations from this study and a few

additional element occurrence records. Grass Mesa has elevated inbreeding, is safe

guarded behind locked gates, and therefore public disturbances are a non-issue making

this population a non-priority for conservation. Of the few populations in this region,

Flat Iron Mesa should be considered as a conservation priority population for the region.

Flat Iron Mesa seems to be the point of incoming gene flow from the Eagle region

(Figure 11), and is therefore critical to maintain connectivity with the rest of *P.*

harringtonii populations. Additional sampling is needed to determine if additional

robust populations are present and to further verify that the region is as unique as it was

found to be in this study.

The RFRV region has four populations that can be utilized to represent the region.

Williams Hill should be targeted for conservation due to its unique rare alleles and as a

representative of the southernmost extent of the region as well as the species. Crown is

the other populations that would need to be conserved and actively management. Crown

is a well-suited representative with high heterozygosity and relatively low levels of

inbreeding and easier to access than the other high elevation site at Light Hill, indicating

that it might encounter a high frequency of anthropogenic effect and therefore should be

managed more intensively. Additionally, initial sampling done at the Cattle Creek Rd

site yielded six individuals for his study, therefore additional sampling is necessary to

determine the viability and size of the population. Initial site assessment for Barber

Gulch was that individuals were robust *P. harringtonii* because exserted stamens were

thought to have been observed on wilting flowers, and a recent element occurrence record

was placed at the site. All individuals at Barber Gulch (BG) were identified as *P.

osterhoutii* (Table 5A) with no indication of admixture or presence of *P. harringtonii*.

Surveys are needed around the Barber Gulch area to determine if there are *P. harringtonii*

populations in the area that could validate the element occurrence record. More surveys

and verification of other element occurrence records should be conducted to better assess

the coverage of the species in the region

Finally, the region east of Glenwood Canyon included that highest levels of

genetic diversity and represents the greatest number of populations for *P. harringtonii*.

The heterozygosity of populations throughout this region are relatively high and

inbreeding levels are all similar. Agnew Gulch, Mayers Gulch and Sheep Creek Uplands

should all be considered for conservation as they were documented to be a critical avenue

for gene flow within the region as well as to other regions (Figure 11). Additionally,

Yarmony should be conserved because it is a critical junction of gene flow between the

NCORV populations and Eagle populations. CO10H9 should be conserved due to

location being in the northern part of the region and range and contain rare alleles that

may be unique to northern climate. These rare alleles may represent unique adaptations

within *P. harringtonii* populations, maintenance of these alleles provides populations the

best chance to effectively respond to climate change. Assisted migration may be

implemented due to shifts in species range and the inability of the species to naturally

shift at the same pace (Williams and Dumroese 2013). If assisted migration is deemed

necessary for this species maintenance of unique genotypes will provide flexibility for

this action. In this study the Eagle area had relatively complete coverage for *P.*

harringtonii, but additional surveys are needed between Burns and McCoy along

Colorado River and from State Bridge to Kremmling along Trough Road to gain a

complete picture of the population extent in the area.

Conclusions

Rare and endemic species, like *P. harringtonii*, are important to maintain

functionality of ecosystems. Focusing on rare and endemic species provides a "litmus

test" for ecosystems and potentially alert management to changing conditions that are not

seen in all organisms within the system of focus. The data presented here documented

the population structure, levels of genetic diversity and the amount of inbreeding

occurring within *P. harringtonii*.

STRUCTURE analysis, PCoA and POPTREEW results suggest that *P.*

harringtonii is composed of three genetic groups, which are analogs to the three

geographic areas where the species is found. GENELAND delineated three additional

populations, Williams Hill (WH), CO10H9 (CH), and State Bridge (SB), which are

divergent from their regional grouping, but this distinction is limited to a single analysis.

Williams Hill and CO10H9 are at the edges of the southern and northern extent of the

range, respectively which may be resulting is slight differentiation due to isolation by

distance. State Bridge is nested in with the other NCORV populations of Yarmony and

McCoy, and shows slight differentiation (Figure 10; cluster 3) with no clear reason for the distinction.

Gene flow between regional groups is high, maintaining continuity of the species across the range. Even though heavy admixture was seen between regions of *P. harringtonii*, it was not the case when *P. osterhoutii* populations were introduced to the data set. *Penstemon harringtonii* and *P. osterhoutii* were determined to be distinct genetic species that coexist throughout the range with minimal to no admixture, despite occupying the same habitat. Additionally, genetic diversity of *P. harringtonii* was high and was not representative of the low genetic diversity commonly seen in rare species. While a few populations did show lower levels of heterozygosity in relation to the bulk of sampled populations, these populations also had elevated inbreeding or low sample sizes which would be a misrepresentation of the population's diversity. These low sample size sites should not be considered for ex-situ conservation actions. Overall, heterozygosity levels of the majority of *P. harringtonii* sampled were similar to common species (Nybom 2004; Kramer et al. 2011) and were higher than other rare Colorado species *Sclerocactus glaucus* (Schwabe et al. 2015) and *Penstemon debilis* (Wolfe et al. 2014).

Populations with low inbreeding and high excepted heterozygosity (Table 3; yellow highlighted) should be targeted by land managing agencies if they are looking to conserve effective genetic signal for *P. harringtonii*. These populations are better equipped to respond to stochastic events because they are not burdened by inbreeding depression nor low genetic diversity. Populations are present within each region that adhere to these parameters. Targeting these populations for conservation within each of the regions will provide effective representation of rare alleles present throughout the

range of *P. harringtonii*. Once populations are identified sufficient number of individuals are needed to collect seeds for grow out and seed banking to ensure seed resources are available for land managers to utilize. *P. harringtonii* produces on average 20 seeds per fruit and 19 fruits per plant according to Neilson (1998) and ideal seed collections from multiple sites within regions following Center for Plant Conservation guidelines. The most robust regional populations should be targeted to account for the bulk of seed and then unique peripheral population collection should be selected to supplement the main collections. Additionally, Williams Hill and CO10H9 both show genetic differentiation from their adjacent regional groups. These populations should be conserved to maintain unique alleles, which may be critical for the potential expansion or shifting in the species range.

Overall, *P. harringtonii* exhibited high heterozygosity and minimal inbreeding, which is promising. Population structure is present with sufficient gene flow to maintain continuity within regions and between them. Management should focus on robust populations from each of the three regions as well as target rare alleles of outlier populations to ensure the greatest diversity for the species is preserved.

CHAPTER III

CHLOROPLAST ANALYSIS

Introduction

Penstemon harringtonii is an endemic species found in the central mountains of Northwest Colorado that is recognized as a Species of Concern or Special Status Species by the U.S. Forest Service and Bureau of Land Management, respectively. Numerous *Penstemon harringtonii* populations are at risk due to increasing oil and gas development, urban and recreational development, and widespread livestock grazing. Land managers need to know how these threats might be affecting specific segments of the species range so that appropriate actions are taken to ensure survival. To better inform land managers, understanding genetic diversity and structure are crucial pieces of information to determine if specific conservation actions are necessary.

Understanding the population structure of a rare plant is vital to ensure that appropriate actions are taken to maintain existing diversity. Phylogeographic investigations have been conducted based on chloroplast DNA (cpDNA) to understand population and regional relationships within single species (Honjo et al. 2004; Yuan et al. 2011) and entire genera (Wolfe et al. 2002; Wolfe et al. 2006). Looking at nucleotide polymorphisms within the chloroplast genome, haplotypes are determined from which individuals can be classified into unique clades with divergent evolutionary histories (Allendorf and Luikart 2009). Previous research utilizing chloroplast genome regions resulted in a better understanding of how *Penstemon* species related to one another,

identifying monophyletic groups but also exposing paraphyletic groups of species that were thought to be closely related (Wolfe et al. 2006). In addition to identifying monophyly, the historic biogeography of genera and species can be determined, as in where specific diversification event occurred. Wolfe et al. (2002) determined where the initial diversification of the tribe Cheloneae, including *Penstemon*, occurred in the Klamath Region of the western United State with subsequent radiation events into the Rocky Mountains and Columbia Plateau. Understanding species adaptive radiation events can also give an inclination of potential future evolutionary trajectory (Losos, 2010), which could aid management in making decision.

Understanding the movement of genetic material across the landscape is important to understanding the dynamics of populations and what factors may be restricting the movement of the species (Falk and Holsinger 1991; Molina et al. 2013). One way for this to be done is through the collection and analysis of chloroplast DNA. This approach gives a historical look at the movement of seeds due to the slow mutation rate of the chloroplast genome and its maternal inheritance, following the seed parent (Falk and Holsinger 1991; Freeland et al. 2011; Molina et al. 2013). Looking at the historic movement of this genetic material can indicate where barriers and corridors might exist. Understanding the barriers throughout the range of a species and how they are affecting gene flow can give land managers tools to target specific regions of the distribution to protect unique diversity and structure.

The maintenance of naturally occuring gene flow pathways between populations can bolster genetic diversity and maintain naturally evolved populations by preserving the evolutionary mechanism (Moritz 1999). Chloroplast data can give land managers a

better understanding of how historical gene flow occurred, so that current populations can

be managed to maintain pre-existing evolutionary units and not anthropogenically-

derived ones (Fraser and Bernatchez 2001). Potential adaptive diversity within

evolutionary units should be maintained by restricting unnatural gene flow between areas

(Moritz 1999). The maintenance and definition of evolutionary units will maintain

diversity within and among regional groups (Crandall et al. 2000) as much as the

evolutionary tracjectory will allow. Barriers and corridors of gene flow need to be

identified as part of the evolutionary process (pollinators) or a result of anthropogenic

activities (livestock movement between regions) to appropriately identify gene flow

patterns to ensure natural evolutionary processes are driving gene flow dynamics. The

data presented here will identify historical gene flow patterns and help determine

potential populations that are crucial to the maintenance of mentioned evolutionary

process within and among populations of *P. harringtonii*.

Anthropogenic activity within the range of *P. harringtonii* has the potential to

alter gene flow among populations by stopping natural processes and/or introducing new

avenues of gene flow. Historical geographical barriers, or lack thereof, may have

allowed for unique populations to form (Irwin and Gibbs 2002). Historical barriers may

have been bypassed or corridors been removed due to anthropogenic activities.

Understanding historical gene flow through chloroplast analysis will allow land managers

to implement management that will conserve populations under the influence of natural

evolutionary processes throughout the range of the species as best various land use plans

will allow for through minimization of threats. The two potential areas of concern for

unintentional gene flow are Rifle and the Roaring Fork River Valley (RFRV). Naturally

occurring geographic barriers isolate these regions, due to an expanse of what is thought to be inhospitable or unoccupied land that separates the populations. The introduction of anthropogenic activities could result in a higher rate of gene flow through recreational and management activities that transfer seeds accidentally between regions or reinforce barriers through anthropogenic activities like oil and gas exploration which disturb continuous native habitat (Trappe et al. 2009; Sertse et al. 2011; Sterling et al. 2012). The data presented here will give some insight to how seeds are moving across the landscape and how disturbance may affect seed movement.

In this chapter, the chloroplast genome of *Penstemon harringtonii* was analyzed to determine polymorphic sites and haplotype diversity throughout its range. Through the analyses of cpDNA, historical patterns in structure and phylogeography will be derived to inform management decisions. Patterns will inform levels of gene flow throughout the range of the species and give indications of how seeds are potentially being transferred within and among populations. Measures of diversity, phylogeography and patterns of gene flow will be used to update management information to help maintain *P. harringtonii* populations.

<div align="center">**Methods**</div>

Extractions

DNA was successfully extracted using a modified cetyltrimethylammonium bromide (CTAB) method that uses the addition of Caylase to break down secondary compounds (Doyle 1987; Friar 2005).

Chloroplast Sequencing

Fifteen general chloroplast specific primers (Shaw et al. 2007) were tested with four individuals from a mix of the populations of *P. harringtonii.* The primers tested were *trnK-rps16x2f2, trnL-rpl32F, rpl32-R-ndhF, trnQ-rps16x1, trnS-trnfM, trnT(GGU)-R-psbD, trnT_tabA-5'trn_tabB, trnV(UAC)-ndhC, atpH-atpI, psbJ-petA, psbE-petL, 5'TrnL(UAA)R-trnT(TabA), trnC-rpoB, psbA-trnH* and *trnS-5'trnG* (Shaw et al. 2007). PCR was carried out 20 µl reactions with 1 µl of genomic DNA (10-20 ng/µl), 1 µl of each primer (10mM), 4 µl of 5X GoFlexi buffer (Promega, Madison, Wisconsin), 1 µl dNTP mixture (2.5 mM; Promega), 1 µl MgCl$_2$ (25mM), 0.3 µl GoFlexi Taq polymerase (Promega), and 10.7 µl of dH$_2$O. PCR amplification was carried out on a Mastercycler proS thermal cycler (Eppendorf, Hamburg, Germany). The reactions were amplified for an initial denaturation at 80ºC for 5 minutes followed by 30 cycles of denaturation at 95ºC for 1 minute, annealing at 50ºC for 1 min with a ramp of 0.3 Cº/s to 65ºC, and a primer extension at 65ºC for 4 minutes, with a final extension step of 5 minutes at 65ºC (Shaw et al. 2007). Products from PCR reactions were verified via electrophoresis using a 1% agarose gel. Of the 15 primer pairs tested, 9 showed positive amplification: *trnL(UAG)-rpl32F, trnQ(UUG)-rps16x1, trnS(UGA)-trnfM(CAU), trnT(UGU)F(TabA)-5'trn(UAA)-R-TabB, atpH-atpI, psbE-petL, 5'trnL(UAA)R-trnT(tabA), psbA-trnH* and *trnS-5'trnG*. 5 µl of amplified PCR products were cleaned utilizing 0.5 µl Exonuclease I (Affymetrix, Santa Clara, CA USA) and 1 µl FastAP, Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific, Lafayette, CO, USA). The mixture was incubated in a Mastercycler proS (Eppendorf) for 15 minutes at 37ºC followed by 15 minutes at 85ºC. Florescent cycle sequencing was performed in 10 µl reactions consisting of 2 µl

5X dilution buffer (Applied Biosystems, Foster City, CA, USA), 0.33 µl BigDye III

(Applied Biosystems), 0.89 µl cleaned PCR product, 0.50 µl primer (1.6pm/µl), and 6.4

µl dH$_2$O. The reactions were amplified on a Mastercycler proS (Eppendorf) at an initial

temp of 96ºC for 1 minute, followed by 30 cycles of 96ºC for 15 seconds, 50ºC for 20

seconds, 60ºC for 4 minutes, and then held at 4ºC.

Cycle-sequence products were analyzed on a 3730XL Genetic Analyzer (Applied

Biosystems) at Arizona State University. Three chloroplast regions were used for in-

depth data collection: *trnQ-rps16x1, psbE-petL,* and *trnS-trnfM*. Both strands of each of

these cpDNA regions were sequenced and assembled in Geneious 8.0.3 (Biomatters

Limited, Auckland, New Zealand). Forward and reverse sequences for each individual of

each region were pairwise aligned by eye, and all sequences were trimmed to a

homologous length.   Consensus sequences were created from alignments for all

individuals from all regions, and all sequences for each individual were concatenated in

the same order: *trnS-trnfM_trnQ-rps16x1_petL-psbE*.

Analysis

DnaSP 5.10.01 (Librado and Rozas 2009) was used is to calculate diversity within

and divergence between *P. harringtonii* populations and various *Penstemon* outgroups.

Populations of *Penstemon harringtonii* were categorized into groups based on

geographical location: Eagle, Northern Colorado River (NCORV), Roaring Fork River

Valley (RFRV) and Rifle. Some populations were removed from the   *Penstemon*

harringtonii dataset due to the microsatellites results (see Chapter II), which indicate that

some samples collected as *P. harringtonii* were actually *P. osterhoutii*. The diversity

statistics reported were number of individuals sampled (N), number of haplotypes (Hp),

haplotype diversity (Hd), nucleotide diversity (Pi) and sequence length (SeqLgth). The

divergence characteristics measured were number of pairwise nucleotide differences

between populations (K_{XY}), the fixation index (F_{ST}), and the average number of

nucleotide substitutions per site between populations (D_{XY}).

MrBayes 3.2.6 (Ronquist et al. 2008) was used to generate Bayesian phylogenetic

trees. Two sets of data were run, one with only *Penstemon harringtonii* individuals and

the second included all individuals sampled for this study. A GTR substitution and

gamma distributed rate variation model was used. A run length of 3,100,000 generations

was used, saving every 1,000[th] tree with a 200,000 iteration burn-in. Consensus trees

were exported to Figtree 1.4.3 (Rambaut 2012) for manipulation.

A haplotype network was generated using PopART (Allan Wilson Centre

Imaging Evolution Initiative; http://popart.otago.ac.nz), with the TCS model (Clement et

al. 2002), where gaps were treated as a 5th state.

Results

A total of 64 *P. harringtonii* individuals sampled from 19 populations were

divided into four geographic regions: Eagle, Northern Colorado River (NCORV),

Roaring Fork River Valley (RFRV) and Rifle. One additional regional group is

designated as East of Glenwood Canyon (E_of_GlenCYN), which is a cumulative

summary of the Eagle and NCORV regions. Outgroups included a total of 27 *Penstemon*

osterhoutii individuals sampled from six populations and two herbarium specimens

which make up POH_outgroup. Three additional herbarium specimens were included as

part of the outgroup data set: *P. secundiflorus, P. angustifolius* and *P. cyathophorus*.

Diversity

Nucleotide diversity statistics within each of the regions of *P. harringtonii* is shown in Table 6 The number of haplotypes (Hp) was greatest in the E_of_GlenCYN region (12) and the lowest in RFRV (2) and Rifle (1). The highest haplotype diversity (Hd) was in the E_of_GlenCYN region (0.833) and POH_outgroup (0.873) and the lowest in the RFRV (0.248) and Rifle (0) regions. The highest nucleotide diversity (Pi) was found in the E_of_GlenCYN region (0.00075) and POH_outgroup (0.00075) and the lowest in the RFRV (0.00010) and Rifle (0) regions. In total 92 individuals were sampled that are delegated to nine groups based on geography or species composition: E_of_GlenCYN, Eagle, NCORV, RFRV, Rifle, POH_outgroup, *P. cyathophorus, P. angustifolius* and *P. secundiflorus*.

Table 6. Chloroplast nucleotide diversity of *P. harringtonii* and *P. osterhoutii*.

Region	N	Hp	Hd	Pi	SeqLgth
E_of_GlenCYN*	43	12	0.833	0.00075	2439
Eagle	35	9	0.765	0.00059	2439
NCORV	8	3	0.607	0.00060	2439
RFRV	15	2	0.248	0.00010	2439
Rifle	6	1	0	0	2439
POH_outgroup	25	8	0.873	0.00075	2439

Number of individuals sampled (N), Number of haplotypes (Hp), Haplotype diversity (Hd), Nucleotide diversity (Pi), and Sequence Length (SeqLgth).
*E_of_GlenCYN indicates all *P. harringtonii* populations that were collected from east of Glenwood Canyon which is the combination of Eagle and NCORV regions.

Pairwise Diversity statistics were calculated between all *P. harringtonii* regions (Table 7). The number of pairwise nucleotide differences between regions (K_{XY}) was the greatest between Eagle and NCORV (5.136) and lowest between Rifle and RFRV (2.133). The highest fixation index (F_{ST}) was between Rifle and RFRV (0.942) and the lowest was between E_of_GlenCYN and RFRV (0.120). The average number of

63

nucleotide substitutions between populations (D_{XY}) was highest among Rifle and

NOCRV regions (0.00201) and lowest between Eagle and RFRV regions (0.00039).

Table 7. Pairwise diversity statistics between *P. harringtonii* regions.

Region 1	Region 2	K_{XY}	F_{ST}	D_{XY}
Eagle	RFRV	2.790	0.134	0.00039
Eagle	Rifle	4.200	0.454	0.00108
Eagle	NCORV	5.136	0.310	0.00110
RFRV	Rifle	2.133	0.942	0.00129
RFRV	NCORV	2.883	0.524	0.00083
RFRV	E_of_GlenCYN	2.808	0.120	0.00047
Rifle	NCORV	4.750	0.737	0.00201
Rifle	E_of_GlenCYN	4.302	0.455	0.00118

Number of Pairwise nucleotide differences between regions (K_{XY}), fixation index (F_{ST}), average number of nucleotide substitutions per site between regions (D_{XY}). NCORV is Northern Colorado River, RFRV is Roaring Fork River Valley and E_of_GlenCYN is East of Glenwood Canyon.

Genetic Structure

The *P. harringtonii* phylogenetic tree is shown in Figure 13. The phylogenetic tree including all samples is shown in Figure 14. Colors given in both phylogenetic trees (Figure 13 and 14) are indicative of which region individuals belong to: Eagle red, NCORV blue, RFRV green and Rifle yellow. The first haplotype network (Figure 15) includes only *Penstemon harringtonii*, while the second haplotype network (Figure 16) includes all collected samples. Mutational steps are represented by hatch marks (including insertions and deletions) and intermediate haplotypes as black filled in circles. The number of individuals assigned to each haplotype of each network is given in Tables 8 and 9 respectively.
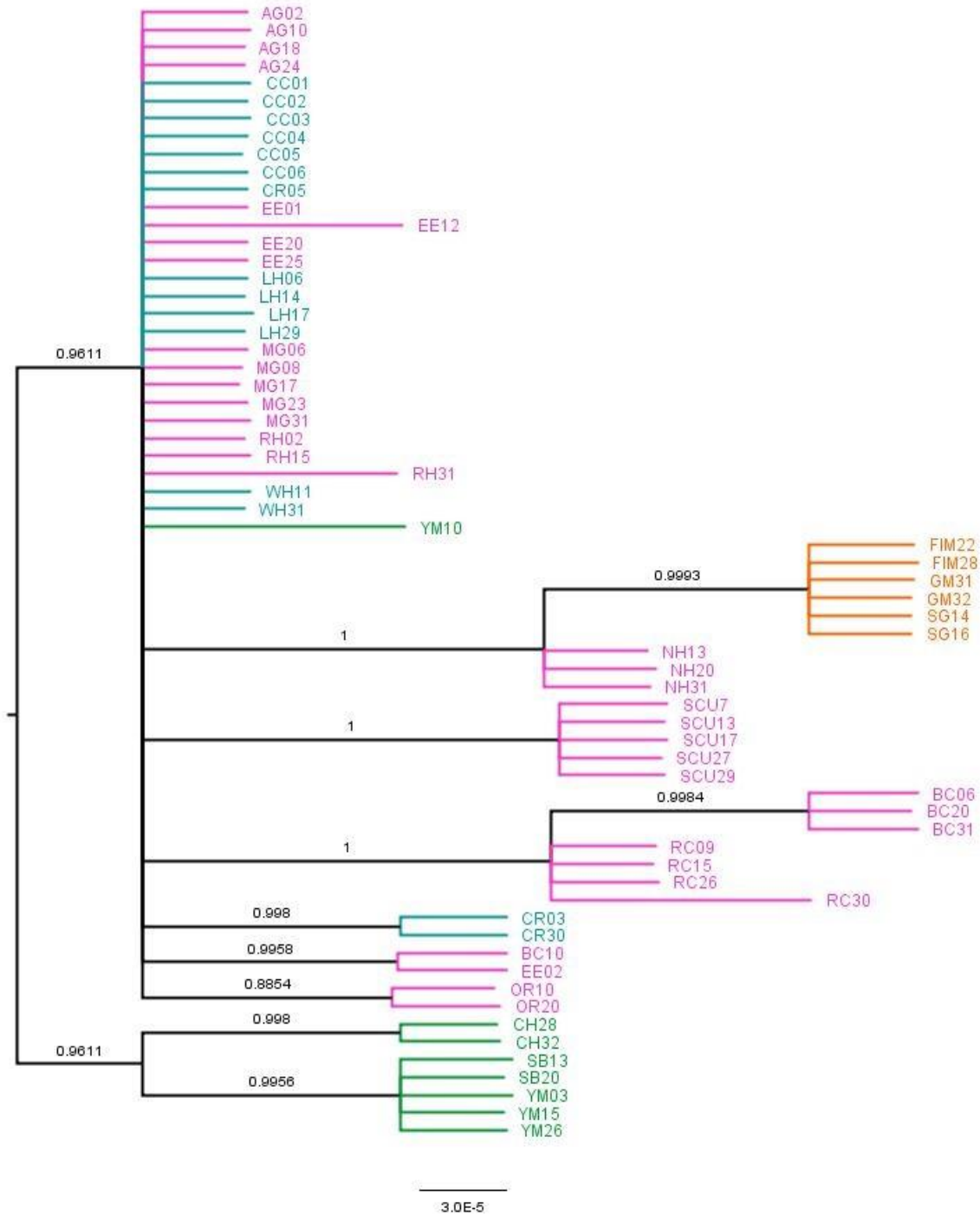
Figure 13. A rooted Bayesian phylogenetic tree for *P. harringtonii* populations.
The tree shows strong support for variation in *trnQ-rps16x1, psbE-petL* and *trnS-trnfM* chloroplast region and posterior probabilities on the branches of the groupings. Eagle (Fuchsia), NCORV (Green), RFRV (Teal) and Rifle (Orange).
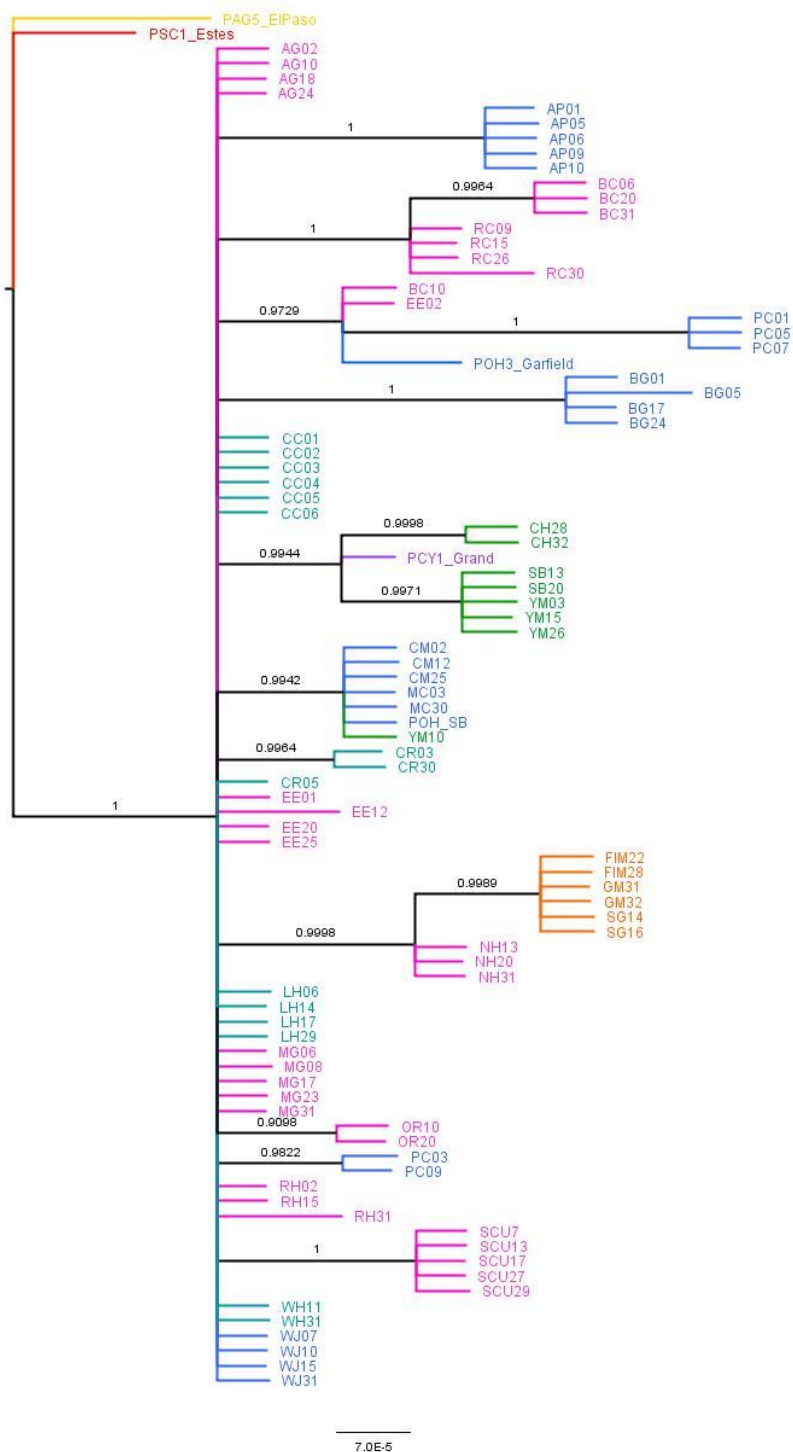
Figure 14. A Rooted Bayesian phylogenetic tree for *P. harringtonii*, *P. osterhoutii*, *P. cyathophorus*, *P. secundiflorus* and *P. angustiflolius* populations.
The tree shows variation in *trnQ-rps16x1, psbE-petL* and *trnS-trnfM* chloroplast region and posterior probabilities on the branches of the groupings. Eagle (Fuchsia), NCORV (Green), RFRV (Teal), Rifle (Orange), POH_outgroup (Blue), *P. secundiflorus* (red), *P. angustifolius* (yellow) and *P. cyathophorus* (purple).
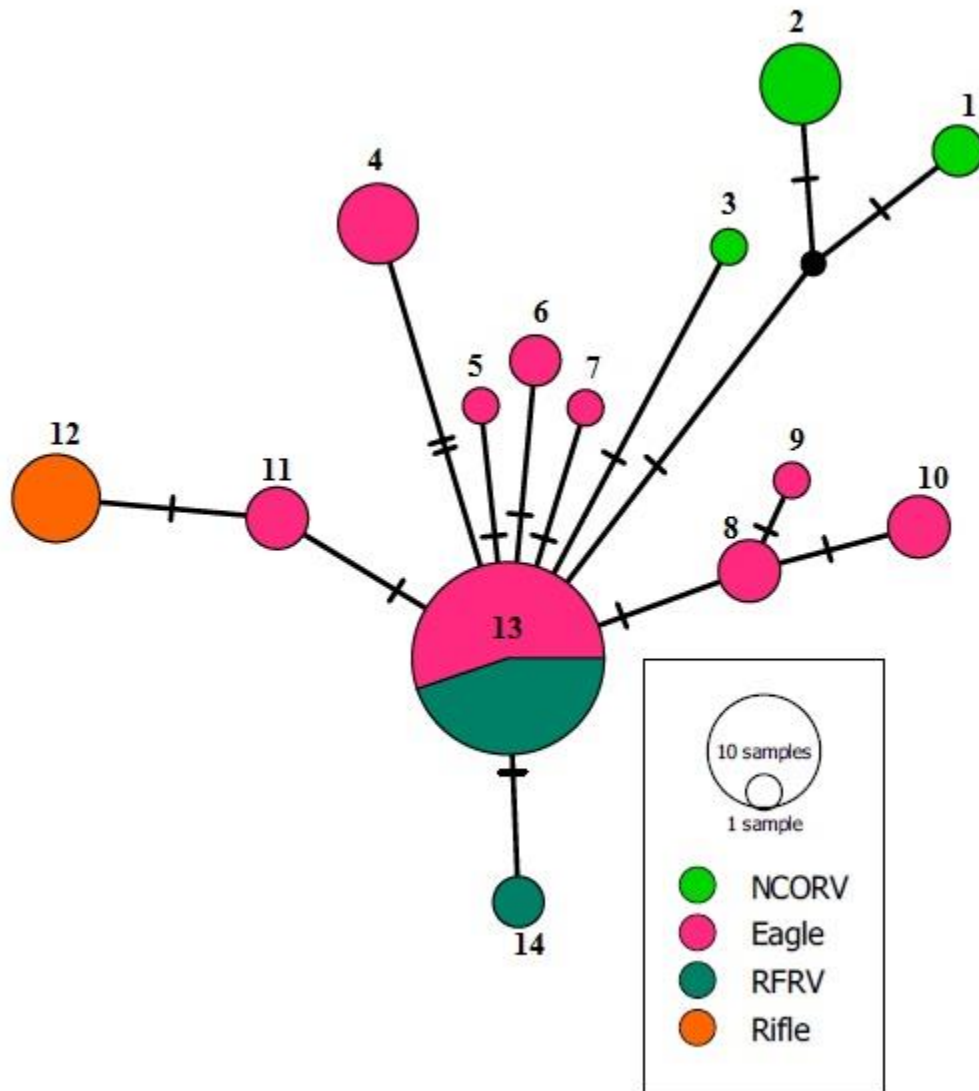
Figure 15. Haplotype network showing variation in the in *trnQ-rps16x1*, *psbE-petL* and *trnS-trnfM* chloroplast region for *P. harringtonii* populations.
Each branch and hatch mark infer a mutational step. Each colored circle represents a haplotype and each black filled circle an inferred haplotype. Populations that make up each haplotype are designated in Table 8.

Table 8. Haplotype identification corresponding to Figure 15, region assignment, number of individuals and population makeup.

Circle Number	Region	N	Population ID
1	NCORV	2	CH
2	NCORV	5	SB, YM
3	NCORV	1	YM
4	Eagle	6	SCU
5	Eagle	1	RH
6	Eagle	2	BC, EE
7	Eagle	1	EE
8	Eagle	3	RC
9	Eagle	1	RC
10	Eagle	3	BC
11	Eagle	3	NH
12	Rifle	6	FIM, SG, GM
13	Eagle/RFRV	29	AG, CC, CR, EE, LH, MG, OR, RH, WH
14	RFRV	2	CR
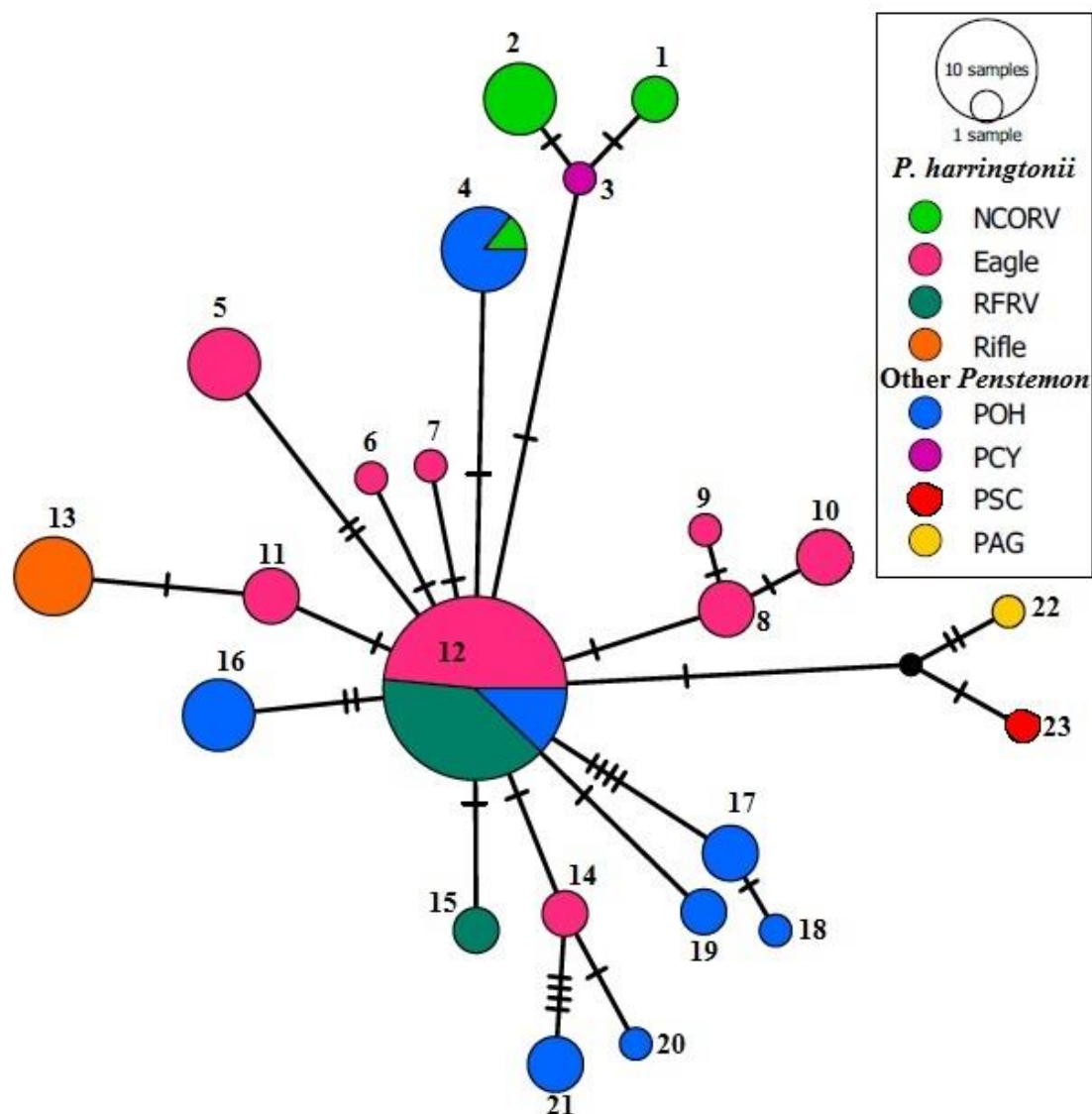
Figure 16. Haplotype network showing variation in the in *trnQ-rps16x1*, *psbE-petL* and *trnS-trnfM* chloroplast region for *P. harringtonii* and the Other *Penstemon* species.
Each branch and hatch mark infer a mutational step. Each colored circle represents a haplotype and each black filled circle an inferred haplotype. Outgroups are designated as the colored circles under the heading of other *Penstemon* in the legend (POH-POH_outgroup, PCY-PCY1_grand, PSC-PSC_Estes and PAG-PAG_ElPaso. Populations that make up each haplotype are designated in Table 9.

Table 9. Haplotype identification corresponding to Figure 16, region assignment, number of individuals and population makeup.

Circle ID	Species	Region	*N*	Population ID
1	*P. harringtonii*	NCORV	2	CH
2	*P. harringtonii*	NCORV	5	SB, YM
3	*P. cyathophorus*	NCORV	1	PCY1_Grand
4	*P. osterhoutii* & *P. harringtonii*	NCORV	7	CM, MC, YM, POH_SB[*P. osterhoutii*]
5	*P. harringtonii*	Eagle	5	SCU
6	*P. harringtonii*	Eagle	1	EE
7	*P. harringtonii*	Eagle	1	RH
8	*P. harringtonii*	Eagle	3	RC
9	*P. harringtonii*	Eagle	1	RC
10	*P. harringtonii*	Eagle	3	BC
11	*P. harringtonii*	Eagle	3	NH
12	*P. osterhoutii* & *P. harringtonii*	Eagle/ RFRV	33	WJ [*P. osterhoutii*], AG, CC, CR, EE, LH, MG, OR, RH, WH
13	*P. harringtonii*	Rifle	29	FIM, SG, GM
14	*P. harringtonii*	Eagle	2	EE, BC
15	*P. harringtonii*	RFRV	2	CR
16	*P. osterhoutii*	Rifle	5	AP
17	*P. osterhoutii*	RFRV	3	BG
18	*P. osterhoutii*	RFRV	1	BG
19	*P. osterhoutii*	RFRV	2	PC
20	*P. osterhoutii*	RFRV/Rifle	1	POH_Garfield
21	*P. osterhoutii*	RFRV	3	PC
22	*P. angustifolia*	Front Range	1	PAG_ElPaso
23	*P. secundiflorus*	Front Range	1	PSC1_Estes

Discussion

Chloroplast genomes are highly preserved due to the role of protein synthesis and involvement in photosynthesis (Lowe et al. 2009). In addition, chloroplast genomes are maternally inherited in most angiosperms (Freeland et al. 2011). Maternal inheritance provides an avenue for identifying seed movement through the interpretation of the chloroplast DNA to better define the mechanism through which gene flow is occurring. Mutation rate within the genome is slow, and mostly composed of single base pair changes or insertion/deletion within non-coding intergenic regions (Falk and Holsinger

1991; Lowe et al. 2009; Freeland et al. 2011). Even with a slow mutation rate, cpDNA provides enough variation to construct phylogenies and identify unique haplotypes through variable single nucleotide polymorphisms or unique insertions/deletions (Lowe et al. 2009; Freeland et al. 2011; Avise 2012). This cpDNA analysis will provide a look at haplotype diversity, a phylogeny from the species and how it relates to outgroups, and give measures of gene flow throughout the range.

Chloroplast DNA analysis for *P. harringtonii* revealed that there is regional based structure and gene flow occurring between all regions maintaining overall diversity within species. Regions east of Glenwood Canyon (E_of_GlenCYN), included a main cluster in the Eagle (Hd = 0.765, Hp = 9) area and the northern part of the Colorado River (Hd = 0.607, Hp = 3) between Kremmling and State Bridge, had the highest haplotype diversity and quantity of unique haplotypes (Table 6). The Northern Colorado River (NCORV) group consists of three unique haplotypes that represent the second highest haplotype diversity of all the regions sampled. The RFRV region shared a majority of haplotypes with the core Eagle region but did have one additional unique haplotype. The Rifle region was grouped together coalescing in a single unique haplotype. Genetic diversity is highest in the regions that are east of Glenwood Canyon and lowest in the isolated Rifle region. Diversity for this region is exceptionally high, 14 different haplotypes, for a rare or endemic plant indicating that *P. harringtonii* does not show low diversity, which is common in rare plants. *Penstemon harringtonii* is classified as rare given its small geographic range and narrow habitat specificity as characterized by Rabinowitz (1981). High diversity within *P. harringtonii* is an indication that the amount

of gene flow within and among regions is high enough to cancel out any detrimental effects of drift that may be occurring within the regions or populations.

Phylogenetic trees are effective at displaying relationships between groups and provide another metric to support regional relationships. The *P. harringtonii* chloroplast phylogeny resolves a clade of the NCORV group minus one individual that falls out with the core group (Figure 13). The North Hardscrabble (NH) population and Rifle area form a clade, with Rifle falling out as its own monophyletic sub-clade, which may indicate descendants of the Rifle populations originated in the Eagle area. Populations at the eastern extent of the Eagle region (Red Canyon and Berry Creek) form a unique clade, while populations closer to the interior of the range clump around the backbone with unique individuals that fall out, but with lower posterior probabilities. For the RFRV, one unique clade is highly supported while the remainder of the region's individuals group with the core group. Common haplotypes being represented from the Eagle and RFRV regions suggest that seed movement among these regions is likely.

Understanding gene flow that is occurring throughout a species' range will aid management in determining appropriate action to secure the persistence of species. Recommendations for populations to be included in Areas of Critical Environmental Concern (ACEC) and developing seed collections strategies which will conserve genetic material, plants and habitat resulting in the perpetuation of the species. Rifle populations are geographically isolated and share a single unique haplotype which could be effectively isolating the populations from the remainder of the regional groups. Rifle, being separated from the core Eagle and RFRV populations indicated by the distinct clade within the phylogenetic tree (orange, Figure 13) and its single haplotype (orange,

Figure 15). The fixation indices (F_{ST}) between Rifle and RFRV, and Rifle and Eagle are 0.942 and 0.454 respectively, indicating high to very high levels of genetic differentiation. These fixation indices are implying that Rifle is descended from the Eagle region and not from the closer RFRV region. These two areas, Rifle and Eagle, show a lower fixation index but are separated by a relatively large distance for genetic material to travel and the upper Sawatch Mountain range potentially acting as a geographic barrier. The case of the relationship between Eagle and RFRV is one of low genetic differentiation (F_{ST}=0.134) even though the two populations are separated by what is thought to be a geographic barrier indicating prevalent gene flow. The barriers between Rifle and Eagle, and Rifle and RFRV, and Eagle and NCORV can be seen in the number of nucleotide differences. Eagle and NCORV is one of the highest of the pairwise comparisons between regions with a K_{XY} value of 5.136 due to the high diversity in both regions, Rifle and Eagle and Rifle and RFRV being on the higher end as well with K_{XY} value of 4.200 and 2.133, respectively (Table 7). The lack of diversity among Rifle and RFRV regions may be resulting in inconsistent K_{XY} values that conflict with fixation index (F_{ST}) values reported earlier. Additional sampling may be needed to improve coverage and better represent genetic diversity within these regions to better support the Eagle to Rifle gene flow, the Rifle descent from Eagle, the degree of isolation in Rifle and the central Rifle-Eagle core premise.

Structure is present within the *P. harringtonii* data set, but when outgroups are introduced, multiple outgroups are nested within *P. harringtonii*. Geographically distant outgroup populations, *P. secundiflorus* from Estes Park, CO and *P. angustiflolius* for El Paso County, CO, form a unique clade within the phylogenetic tree (Figure 14) and two

distinct haplotypes within the haplotype network (Figure 16), representing the Front

Range. The same *P. harringtonii* relationships hold when outgroups were introduced

with *P. osterhoutii* and *P. cyathophorus* integrated among the *P. harringtonii* samples.

Penstemon osterhoutii consisted of numerous unique haplotypes, where haplotype

diversity (Hp = 0.873) was similar to that of the core *Penstemon harringtonii* group

(E_of_GlenCYN; Hp = 0.833; Table 6). The Prince Creek *P. osterhoutii* population is

split into two distinct clades/haplotypes while the Anvil Points *P. osterhoutii* population

forms a distinct clade/haplotype. The *P. osterhoutii* populations at McCoy (MC) and

Catamount (CM) that were initially collected as *Penstemon harringtonii,* form a unique

clade/haplotype that also includes a *P. osterhoutii* herbarium specimen and an odd

Yarmony (YM) individual, which according to microsatellite data is *P. harringtonii*.

This unique clade formation supports what the microsatellite data found via

STRUCTURE analysis, Catamount and 20 individuals from the McCoy population

grouped with all other *Penstemon osterhoutii* populations (Chapter 2, Figure 5A).

Mutational steps of *P. osterhoutii* samples from the central haplotype don't exceed four

steps except for a subset of the Prince Creek individuals and Barber's Gulch individuals,

which both show five or more step from the central *P. harringtonii* haplotype. Due to the

integration of the outgroup species, *Penstemon harringtonii* is not monophyletic, nor is *P.*

osterhoutii, which indicates recent divergence between the *P. harringtonii* and the

outgroup species. Finally, the Eagle and RFRV regions share the most prominent

haplotype (circle 13, Figure 15) and create an admixed group of individuals on the

backbone of the phylogenetic tree (pink and teal, Figure 13). A shared haplotype across

two regions like this could be a result of ongoing gene flow, an artifact of historical gene

flow, or the representation of an imminent divergence event resulting in unique genotypes.

Wolfe et al. (2006) used two noncoding chloroplast intergenic spacer regions, *trnT-L* and *trnC-D,* and nuclear ribosomal internal transcribed spacer (nrDNA ITS) regions to examine phylogenetic relationships over a large range of species within Plantaginaceae. Wolfe et al. (2006) placed *P. harringtonii* in a clade with *P. saxosorum*, *P. mensarum* and *P. bicolor* based on the cpDNA, while *P. harringtonii* was unresolved along a basal branch of the local clade that included several species utilized as outgroups in my cpDNA analysis. The cpDNA phylogenetic trees displayed *P. secundiflorus* and *P. angustifolius* in neighboring clades with *P. harringtonii* being unresolved. The ITS data set from Wolfe et al. (2006) included all of the representative outgroups and their relative relationships to *P. harringtonii*. Overall, the Wolfe et al. (2006) phylogenetic trees from nuclear and cpDNA did not provide support for the placement of *P. harringtonii* nor most of the surrounding species and clades due to a bootstrap value of 70% or less. Of the outgroup species represented in the Wolfe et al. (2006), none were ever sister taxa to *Penstemon harringtonii* but there was limited support for this as well, due to low bootstrap values. Wolfe et al. (2006) results were based on very few individuals for each species, as a result the findings are used as a stepping off point for other investigation to validate or refute the clade formation reported by Wolfe et al. (2006). Due to the limited sample size and large scope of the study, the relationship between *P. harringtonii* and closely related species is still largely unresolved.

Microsatellite data outlined in chapter II, as shown in the STRUCTURE analysis (Figure 5A) effectively separated out the populations that were misidentified in the field

as *P. harringtonii* and are suspected to be *P. osterhoutii* based on the grouping. This result is supported further with the chloroplast analysis of all collected population samples and is seen in the phylogenetic tree (Figure 14) and the haplotype network (Figure 16) where populations of Catamount (CM) and McCoy (MC) group with POH_SB, which was a Denver Botanic Garden specimen that was propagated from wild collected seed near State Bridge, CO, which is in the close vicinity of CM and MC populations. Nuclear data also indicated that Barber's Gulch (BG) and Wingo Junction (WJ) were *P. osterhoutii*. In the phylogenetic tree (Figure 14) and haplotype network (Figure 16) BG is grouping only with itself with no indication of a relationship with any *P. osterhoutii* samples that were included in the analysis, and were five mutational steps away from the next haplotype, which is the central *P. harringtonii* haplotype. This may indicate that BG is more closely related to *P. harringtonii* than to *P. osterhoutii,* but due to the fragmented representation of *P. osterhoutii* this conclusion has little support. A local representative of *Penstemon osterhoutii* may provide the linkage of BG to *P. osterhoutii* to better support the notion of recent divergence between *P. harringtonii* and *P. osterhoutii.* The Wingo Junction population is a bit more perplexing due to the minimal admixture seen in the STRUCTURE diagram assigning it to the *Penstemon osterhoutii* microsatellite genotype, while the cpDNA analysis classifies it as *P. harringtonii* by sharing the central haplotype.

Conclusions

Geographic groups were well represented in the results of the cpDNA analysis with each region having one or more representative haplotypes. Overall, the cpDNA is providing support of a central core of diversity within the interior and greater distinction

or structure at the periphery of the range. The Eagle region has the highest genetic diversity and shares similar haplotypes with the RFRV, which has one additional unique haplotype, while Eagle has several unique haplotypes. The central core that is focused in the Eagle region makes the area a potential area of conservation priority. The NCORV region is of potential conservation importance for land management agencies due to the diverse haplotypes. The Rifle region, due to its location on the western edge of the range, significant isolation from other regions and the prevalence of disturbances in the area, is of concern and should be considered for additional safeguards that are in accordance with the current land use plan. Additionally, there should be more samples analyzed for the Rifle region to provide an assured understanding of the haplotype diversity and overall uniqueness of the region. Also, surveys and sampling to see if there are any transition populations between the known populations in NCORV and the Eagle region, will give a better determination of the current status of gene flow between the two regions. The data from chloroplast analysis of *Penstemon harringtonii* shows that (i) a few sampled populations were misidentified, which was also supported by nuclear microsatellite data (Chapter II) (ii) NCORV and Rifle regions show high F_{ST} values when compared to the core group of individuals in the Eagle region, indicating a pronounced level of genetic differentiation and potential lack of, or reduced rate of gene flow between the regions, (iii) high levels of genetic diversity in core regions of Eagle and RFRV and (iv) *P. harringtonii* and *P. osterhoutii* are recently diverged and not monophyletic.

CHAPTER IV

SUMMARY

Introduction

Penstemon harringtonii is a rare endemic species of central Colorado within the sagebrush steppe and similar habitats in the region. This species is on the Bureau of Land Management (BLM) State Director's Sensitive species list and the U.S. Forest Service Regional Forester's Sensitive Plant Species List. The habitat where *P. harringtonii* occurs is under threat from numerous anthropogenic activities, oil and gas development, livestock grazing, and recreation, resulting in negative pressures on the persistence of the species.

Penstemon harringtonii populations are separated into three disjunct regions. These three regions are the areas around the community of Rifle, the Roaring Fork River Valley and areas east of the Glenwood Canyon along the I-70 corridor to Edwards and north to Kremmling. A better understanding of the populations within these regions and how they relate other regions is necessary to effectively manage the species. Previous genetic studies of Plantaginaceae (Kramer 2002) have been able to effectively delineate groups at the Tribe level of taxonomic classification with good support, but when trying to species delineate within *Penstemon* a consensus relationship is non-existent (Kramer 2006). Efforts have been made to better define the relationships within *Penstemon* through the development of genus level genetic markers (Wessinger et al. 2016; Dockter et al. 2013; Kramer et al. 2011; Kramer and Fant 2007). In utilizing these markers

biologist are gaining further understanding of how individuals of *Penstemon* species are interacting within and among populations (Wolfe et al. 2016; Wolfe et al. 2014; Kramer et al. 2011), and how landscape features (Wolfe et al. 2014; Kramer et al. 2011) and pollinators (Kramer et al. 2011) affect gene flow and population structure. *Penstemon harringtonii* has minimal analysis completed for it, but Neilson (1998) did an investigation of the breeding biology and ecology of *P. harringtonii*. These analyses provided some understanding of pollinators and seed production, but lacked further analysis of the overall status of the species. Genetic investigations, as mentioned above, will provide valuable information to fully understand this species status.

 Penstemon harringtonii is of interest due to the large number of occurrences that are present on Bureau of Land Management lands in close proximity to oil and gas facilities, recreational sites and within grazing allotments. The species receives special considerations under current land use plans, but to ensure that the appropriate management is occurring additional information is needed across the entire range. The range wide understanding was accomplished by investigating the chloroplast genome and microsatellite regions of the nuclear genome to determine genetic diversity, levels of gene flow, population structure and to determine if the landscape is impacting the level of differentiation between regions. This information is utilized to formulate conservation recommendations that will be made available to land management agencies. The specifics of this genetic investigation of *Penstemon harringtonii* are as follows: (a) relationship between *P. harringtonii* and other *Penstemon*, (b) *P. harringtonii* population structure and differentiation, (c) levels of genetic diversity, gene flow and inbreeding for the populations of *P. harringtonii*.

Genetic Relationships of
Penstemon harringtonii
and Other *Penstemon*

In order to determine the status of *P. harringtonii,* its relationship to other

Penstemon species within its range needed to be verified. The two species of general

interest were *P. cyathophorus,* because Penland (1958) mentioned it as the closest related

species when identifying *P. harringtonii,* and *P. osterhoutii*, because of the substantial

overlap in ranges and similarities in morphology with *P. harringtonii*. Three regions

from the chloroplast genome and nine microsatellite markers were analyzed to determine

the relationship between *P. harringtonii* and the other focal *Penstemon* species.

In Chapter 3, chloroplast DNA analysis showed a lack of monophyly for the

species of interest (Figure 14). Some individuals of *P. harringtonii* and *P. osterhoutii*

had more affinity for each other when in close proximity than to conspecifics in other

regions. *Penstemon cyathophorus* grouped with *P. harringtonii* populations in the

northern extent of the range instead of forming a unique group. This lack of monophyly

among species indicates recent divergence within the genus and between these three

species. Further supporting the patterns of unresolved or minimally supported species

relationships within *Penstemon* that Wolfe et al. (2006; 2002) reported. To better

understand the relationship between *P. harringtonii* and *P. cyathophorus* additional

samples are needed.

In Chapter 2, microsatellite analysis further explored the relationship between *P.*

harringtonii and *P. osterhoutii* through the utilization of nuclear microsatellite markers.

Microsatellite analysis utilized nine variable loci to better determine the relationship

between *P. harringtonii* and *P. osterhoutii*. Using the model based clustering software

STRUCTURE and STRUCTURE HARVESTER the results showed clear distinction of

P. harringtonii populations from *P. osterhoutii*. Minimal to no introgression between the

two groups that STRUCTURE created was present (Figure 5A), indicating lack of

hybrids and hybridization. Further supporting this is the example at McCoy (MC), where

samples were collected as *P. harringtonii* but a portion of them were genetically

identified as *P. osterhoutii*. Looking at this population in Figure 5A, no introgression is

seen, further supporting the notion that these two species are distinct but also confirming

that in addition to having overlapping ranges, they also have overlapping population.

The analysis of the chloroplast DNA and the microsatellite analysis provides the

necessary support to show that this species is distinct. The distinction is well supported

by the microsatellite analysis in chapter 2 and the STRUCTURE analysis. The

chloroplast DNA analysis supports that the divergence of *P. harringtonii* was relatively

recent due to the lack of phylogenetic monophyly. These analysis will provide

management the necessary support to manage *P. harringtonii* as a distinct species.

<div align="center">

***Penstemon harringtonii* population**
Structure and Diversity

</div>

The distinctiveness of *P. harringtonii* supports the principle that the species

would have regional genetic structure. The Chapter 2 microsatellite STRUCTURE

analyses indicate that *P. harringtonii* has three distinct regional genetic groups: Rifle,

Roaring Fork River Valley and East of Glenwood Canyon (Figure 7B). The three regions

are further supported by the principle coordinates analysis (Figure 8), GENELAND

analysis (Figure 10) and phenogram (Figure 12). The Rifle region represents the

westernmost extent of the range. The Roaring Fork River Valley is in the center of the

range with representatives at the highest elevations, the southernmost extent, and one

population containing rare alleles (Williams Hill). The East of Glenwood Canyon regions encompasses the greatest number of populations, and the eastern and northernmost populations. These three regions represent the population structure based on microsatellite allelic data. Genetic differentiation among regions showed low to medium levels of distinctiveness. The GENELAND analysis recognized the three regions as well as two unique populations (Williams Hill and CO10H9), which contain unique alleles.

Heterozygosity, inbreeding coefficients and gene flow were all at acceptable levels. Heterozygosity was exceptionally high and inbreeding was well below 0.5 for all populations. The number of migrants was over one for all pairwise comparisons within and among regions, supporting a high level of gene flow. Gene flow was graphically represented in a minimum spanning tree (Figure 11) which was completely connected, indicating that genetic material is moving in or out of every population to some degree. Agnew Gulch, Mayers Gulch, Sheep Creek Uplands and Yarmony are critical avenues of gene flow within the East of Glenwood Canyon region. Crown and Flat Iron Mesa are critical for gene flow into or out of the Roaring Fork River Valley and Rifle regions, respectively.

The microsatellite analyses indicate that *P. harringtonii* has high diversity across its range and adequate gene flow to maintain continuity between and among regions. Specific populations within each region were determined to be critical to maintain gene flow within populations and some critical to maintain connectivity between the regions. Additionally, unique populations were identify that harbor rare alleles that further

insulate the resilience of *P. harringtonii*. High diversity and gene flow levels throughout the range indicates that *P. harringtonii* is a cohesive and resilient species.

Pollinators and Gene flow

The high levels of gene flow that is occurring within and among regions of *P. harringtonii* leads to the question, how? Neilson (1998) investigated the pollinator and breeding ecology of *P. harringtonii,* and established that the dominant pollinators were bees from the Megachilidae family, specifically the genus *Osmia,* and *Pseudomasaris vespoides* from the family Vespidae, subfamily Masarinae. *Pseudomasaris vespoides* is thought to preferentially choose *Penstemon* species over other species when resource are abundant, especially those species with larger throat openings and flowers (Tepedino 1979; Cooper 1952). *Pseudomasaris vespoides* is thought to be a *Penstemon* specialist within Colorado, with the utilization of other species as necessary for survival (Cooper 1952), but elsewhere may utilize a wider range of species at higher rates (Tepedino 1979). Species of *Osmia* are specialist of *Penstemon* (Crosswhite and Crosswhite 1966), and can be solely dependent on a single *Penstemon* (Crosswhite and Crosswhite 1966) or utilize *Penstemon* and other species as a source of pollen and nectar (Lewisohn and Tepedino 2007; Tepedino et al. 1999; Crosswhite and Crosswhite 1966). The two most prevalent pollinators of *Penstemon* and *P. harringtonii* are medium sized insects, and therefore this put limits on distance traveled for pollination.

Osmia bee species and *P. vespoides* are the two main groups that have been observed visiting *Penstemon* species (Lewisohn and Tepedino 2007; Tepedino et al. 1999; Tepedino 1979; Crosswhite and Crosswhite 1966; Cooper 1952) and *P. harringtonii* (Neilson 1998). These species are solitary insects that nest above and below

the ground (Crosswhite and Crosswhite 1966; Cooper 1952) and forage around an established nest (Guedot et al. 2009), limiting the distance these pollinators travel. Determining how far the most popular pollinators will travel to gather resources will explain whether they are the source of gene flow between regions. According to Greenleaf et al. (2007), body size is directly correlated to the foraging range of bees, which Guedot et al. (2009) validated by utilizing it on *Osmia* species. Guedot et al. (2009) determined the greatest distance that the largest *Osmia* species would travel was 1.8 km. Greenleaf et al. (2007) looked at several *Osmia* species as well, which reported foraging ranges between 0.5 and 3 km. Based on reported foraging distances of *Osmia* species the likelihood that gene flow among *P. harringtonii* regions is occurring due to foraging behavior is low. Due to the solitarity nature of these bees, they are highly mobile moving between suitable nesting habitats (Torné-Noguera et al. 2014) and they could potentially follow resources as flower senescence occurs throughout the season. This could potentially explain a transition of pollinators from low elevations, where plants flower early, to higher elevations, where plants flower later. If pollinators show special affinities for specific species (Crosswhite and Crosswhite 1966) then the drive to follow the preferred resources would be high enough to drive gene flow between regions as well.

The most prominent pollinators may not be the pollinators that are effectively moving genetic material between *P. harringtonii* regions. Pollinators that are seen visiting the plant less often or not at all according to Neilson (1998) may be the critical pollinator for long distance gene flow. Two pollinators to consider that Neilson (1998) observed are *Anthophora bomboides* and *Bombus appositus,* which both are larger in size

and generalists. Looking at similar *Anthophora* and *Bombus* species within the Greenleaf et al. (2007) study, the calculated foraging range is slightly elevated as compared to *Osmia* species with a potential range of 10 km for *Bombus* species (Pasquet et al. 2008).

Finally, pollinators that have not be observed visiting *P. harringtonii* but could still be a pollinator need to be considered as a potential avenue for long distance gene flow. The Greenleaf et al. (2007) analysis indicated that large carpenter bees, *Xylocopa* species, have been recorded to forage in excess of 10 km from a nest site (Pasquet et al. 2008; Greenleaf et al. 2007). The floral opening of *P. harringtonii* is large enough to provide the necessary "landing pad" for a larger bee. Alternatively, the critical pollinators could be birds, which could easily connect the regional groups of *P. harringtonii* without needing to be a major component of the species pollination ecology. *Penstemon harringtonii* flowers are long with an ampliate-funnelform throat and two exserted stamens (Penland 1958), which are both characteristics associated with transitional bird pollination syndromes (Lara and Ornelas 2008; Crosswhite and Crosswhite 1982). In addition to the morphology of *P. harringtonii,* the variability of color from purple to light and dark pink leads to the thought that a transition of pollination syndrome could be occurring, similar to *Penstemon roseus* (Lara and Ornelas 2008). According to Clements (1923), *Selasphorus rufus* (Rufous hummingbird) visited *Penstemon gracilis* and *P. secundiflorus,* which are both bee pollinated and have long slightly tubular corollas and are light purple and pink, respectively. The fact that *P. harringtonii* has a narrower tubular corolla and exserted stamens indicates that it may be slightly more adept to successfully be pollinator by a hummingbird.

Gene flow within *P. harringtonii* indicates that genetic material is being effectively transferred between and among the regions. The increased levels of gene flow doesn't align with the behaviors and capabilities of the main pollinators for *P. harringtonii*. To account for the levels of gene flow present the potential for a larger bee or bird pollinator is a possible explanation to investigate further. Overall, the current knowledge of pollinator ecology for *P. harringtonii* is still lacking and needs additional resources allocated to effectively determine how pollinators a influencing the species.

Conservation Recommendations

Land management agencies need to have the appropriate information to effectively take steps to maintain the persistence of *Penstemon harringtonii*. The first step in that process is to recommend which of the populations examined in this investigation would warrant conservation priority (Table 10) and acknowledge that additional sampling of populations will need to be completed to address areas that were not included in this study. Second, it is important to discuss threats and additional actions that would further support the persistence of the species. Methods to conserve *P. harringtonii* populations can vary greatly depending on the time and resources available. Here I provide a summary of the genetic status of *Penstemon harringtonii* and recommendations of (1) specific protections of unique populations, (2) guidelines for expansion of monitoring programs and (3) an overview of the implementation of an ex-situ seed collection program. These three recommendations are methods that fall along the spectrum of highly involved to least involved, and require substantial to minimal resources to complete, giving management agencies flexibility is how they manage this species.

Table 10. Genetic diversity statistics for populations of *P. harringtonii* that are being recommended for conservation priority.

Population	Region	N	N_a	N_e	H_o	H_e	F_{IS}
Agnew Gulch	Eagle	32	10.889	6.364	0.613	0.713	0.114
Sheep Creek Uplands	Eagle	32	11.222	6.526	0.594	0.757	0.201
Mayer Gulch	Eagle	32	11.778	7.455	0.603	0.761	0.194
CO10H9	NCORV	33	8.111	4.831	0.572	0.742	0.217
Yarmony	NCORV	32	10.444	6.124	0.572	0.732	0.196
Crown	RFRV	32	8.333	4.601	0.602	0.714	0.158
Williams Hill	RFRV	32	5.222	2.921	0.532	0.585	0.126
Spruce Gulch	Rifle	32	8.444	4.671	0.604	0.676	0.094
Flat Iron Mesa	Rifle	32	8.333	4.382	0.637	0.683	0.066

I recommend protection of the populations listed in Table 10, based on maintaining genetic diversity, populations that are thought to perpetuate the species genetic signal, and populations with unique genetic signal. The first six populations in Table 10 make up the representative populations for the East of Glenwood Canyon region. Agnew Gulch, Sheep Creek Uplands and Mayer Gulch were selected due to their role in gene flow throughout the East of Glenwood Canyon region. These three populations represent geographic and genetic junctions among certain parts of the region: Mayers Gulch is the junction that connects the East of Glenwood Canyon region to the other two regions, Agnew Gulch is the junction to a majority of the populations that make up the Eagle sub-region, and Sheep Creek Uplands is the junction point that connects the north (NCORV from Burns to Kremmling) and south (Burns down into the I-70 corridor from Edwards to Dotsero) constituent parts of the East of Glenwood Canyon region. CO10H9 and Yarmony were selected because of unique characteristics as characterized in the GENELAND analysis which will provide a pool of unique alleles, further adding to the robustness of the species as a whole and was a critical junctions for gene flow,

respectively. Within the Roaring Fork River Valley region Williams Hill and Crown were both selected as are critical junction points for the movement genetic material in or out of their particular region and for the contribution of unique alleles to the gene pool that are key to the persistence of the species. Finally, the Spruce Gulch and Flat Iron Mesa populations were selected for being another critical junction for the flow of genetic material into the Rifle region and to have an ample amount of diversity represented in that region.

To lessen impacts on regions or populations under high levels of disturbance, immediate conservation action may be necessary to ensure the persistence of *P. harringtonii* in the area. The Rifle region has extensive oil and gas production occurring throughout, cattle grazing and a minor component of recreation present. Oil and gas development has the potential to destroy areas of habitat critical to pollinators and other unknown effects on pollinator behavior in the presence of high anthropogenic disturbance (Hadley and Betts 2012). Cattle grazing is present as well, and has a more localized impact to a population, with disturbances localized around and along water sources (DeYoung 2017). A slight component of recreation is present and may be of concern if trail usages increase and rogue trail building becomes more prevalent. The overall status of the Rifle region is one of concern due to the small number of populations and the high incidence of disturbances, which pose an immediate threat to the long term persistence of *P. harringtonii* within the area. Protecting these populations or a subset is critical to maintaining the full extent and variability of the species. Designation of an Area of Critical Environmental Concern (ACEC) somewhere within the Rifle region would provide necessary protections for *P. harringtonii*. Based on the genetic data, an ACEC

that encompasses the Flat Iron Mesa sampling site and as much of the surrounding area as possible would be advised. An ACEC would conserve the components of the Flat Iron Mesa population, which is critical to gene flow into and out of the region, but is also situated near numerous occurrence records indicating the potential to protect a large number of individuals. An ACEC could also be utilized for additional research into *P. harringtonii* by allowing oil and gas operations to continue while establishing long term trend monitoring throughout the ACEC to assess the species response to disturbance. In addition to oil and gas disturbance, fence exclosure experiments could also be implemented to determine the effect of cattle grazing disturbance as well.

Trend monitoring of *P. harringtonii* can be implemented to assess the response of the species to various weather conditions and to monitor the growth, decline or stability of the species. Several methods of monitoring can be implemented so that it can be scalable to the level of resources available. A recommendation of installing four weather stations placed at populations from the two East of Glenwood Canyon sub-regions NCORV and Eagle, RFRV and Rifle regions would provide weather data across the range of the species (e.g. populations at State Bridge, Agnew Gulch, Flat Iron Mesa and Cattle Creek Road). Demographic monitoring plots could be established in close proximity to weather data collection sites to provide correlative data. The demographic data that could be collected is: flowering success (yes or no), inflorescence size (number of flowers) and density (number of flower/length), overall habit of individuals (height and basal rosette diameter), and number of individuals per transect. These monitoring plots would ideally be permanent plots with permanent sampling units. Sample size equation 3 (Elzinga et al 1998) would be used to determine appropriate number of

transects if plots were established. Additionally, four long term trend monitoring plots could be set up within the four regions to further assess overall stability of species and provide an additional option for monitoring that is less time consuming than the demographic protocol (recommended site locations for long term trend plots: CO10H9, Mayers Gulch, Spruce Gulch, Crown). These initial four plots would measure mean plant density and utilized sample size equation 3 (Elzinga et al 1998) as well. This monitoring would provide a robust representation of the overall health and status of the species across its range.

In addition to long term trend monitoring, additional surveys for *P. harringtonii* are necessary to complete the understanding of the species. The East of Glenwood Canyon region needs to be surveyed to determine if substantial populations exist along the Colorado River between the McCoy and Sheep Creek Upland populations and along Trough Road between State Bridge and Highway 9. Within the Roaring Fork River Valley region additional sampling near Barbers Gulch, Smith Gulch, and Prince Creek will provide a better representation within the area and provide validation of nearby element occurrence records. Sampling around the Basalt and Wingo areas and a more robust collection at the Cattle Creek Road population would provide a better understanding of *P. harringtonii* in the RFRV region. These additional collections would provide missing information in the understanding of *P. harringtonii* across its range.

The final recommendation, seed collection, is to be implemented to hedge against stochastic population loss and species extinction. The Center for Plant recommendations are the accepted method of how to collect seeds of rare, threatened or endangered plants. The guidelines are as follows: collect no more than 10% of seeds from an individual,

collect from 50 individuals within a populations when the populations are greater than 50, otherwise collect from all available and unbiased collections are ideal (Guerrant et al 2014; Raven et al. 2013). *Penstemon harringtonii* has elevated genetic diversity therefore samples need to be taken from genetically unique populations to best represent the extent of the diversity across the range of the species (Guerrant et al 2014). Since there is an understanding of genetic structure, population collections can fully represent the diversity within the resulting seed collection. Seed collections provide management a resource to utilize in the case of catastrophic events that result in a severe reduction in population number. Reintroduction of a species would be in response to a catastrophic anthropogenic event that results in unnatural loss of individuals, so a course correction of an addition of seeds would allow the evolutionary processes to occur and respond in a natural context effectively allowing the population to recover with minimal management interaction (Maschinski et al. 2012). Seed collections of *Penstemon harringtonii* should be collected from as many populations, within each region, as possible. This collection will be a genetically accurate representative of *P. harringtonii* that will provide management an additional tool to ensure the persistence of the species.

  Penstemon harringtonii is scattered throughout north central Colorado in three disjunct regions and land managers needed to know the extent at which the species was connected between the regions. The overall status of the species was in question, diversity levels and inbreeding values were needed to better assess how the species was doing, and the relationship with a morphologically similar species of *Penstemon osterhoutii* was unclear. Using genetic tools, *P. harringtonii* and *P. osterhoutii* were determined to be distinct species and the divergence among Penstemon species is

relatively recent. *Penstemon harringtonii* has high levels of genetic diversity and low levels of inbreeding across all populations sampled and ample gene flow occurring within and among populations and regions. The conclusions of this genetic investigation and the conservation recommendation provides land management agencies sufficient evidence to better evaluate the future of *Penstemon harringtonii*.

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