May 2023

The Effects of Habitat Isolation on Fine-Scale Genetic and Geographic Structure of Populations of Two Threatened Endemic Insects in Southern Nevada, Pseudocotalpa giulianii and Icaricia shasta charlestonensis

Kristen Tovar

THE EFFECTS OF HABITAT ISOLATION ON FINE-SCALE GENETIC AND GEOGRAPHIC STRUCTURE OF POPULATIONS OF TWO THREATENED ENDEMIC INSECTS IN SOUTHERN NEVADA, *PSEUDOCOTALPA GIULIANII* AND *ICARICIA SHASTA CHARLESTONENSIS*

By

Kristen Tovar

Bachelor of Science – Marine Biological Sciences
University of Hawaii at Manoa
2015

A thesis submitted in partial fulfillment
of the requirements for the

Master of Science – Biological Sciences

School of Life Sciences
College of Sciences
The Graduate College

University of Nevada, Las Vegas
May 2023

UNLV | GRADUATE COLLEGE

Thesis Approval

The Graduate College
The University of Nevada, Las Vegas

January 13, 2023

This thesis prepared by

Kristen Tovar

entitled

The Effects of Habitat Isolation on Fine-Scale Genetic and Geographic Structure of Populations of Two Threatened Endemic Insects in Southern Nevada, *Pseudocotalpa giulianii* and *Icaricia shasta charlestonensis*

is approved in partial fulfillment of the requirements for the degree of

Master of Science – Biological Sciences
School of Life Sciences

Daniel Thompson, Ph.D.
Examination Committee Chair

Donald Price, Ph.D.
Examination Committee Member

Allen Gibbs, Ph.D.
Examination Committee Member

Margaret Odlum, Ph.D.
Graduate College Faculty Representative

Alyssa Crittenden, Ph.D.
Vice Provost for Graduate Education & Dean of the Graduate College

Abstract

Assessing the role geographic isolation and ecological specialization have on phylogeographic patterns contributes to our understanding of the evolutionary history of a species and the processes that erode genetic diversity. I used mitochondrial and nuclear genomic sequences to assess whether habitat isolation has shaped the fine-scale patterns of present-day genetic structure and diversity in two threatened insect species endemic to southern Nevada. *Pseudocotalpa giulianii* (Coleoptera; Scarabaeidae) is a dune obligate scarab beetle endemic to only two small, isolated sand dunes in Nye County, Nevada, with a usable habitat of less than 4.2 km sq that is impacted by on-going degradation. Analysis of the pattern of divergence of five mitochondrial DNA haplotypes revealed a high level of divergence between the two sand dunes, separated by only 7 km of inhabitable desert (Fst: 0.077, Nm: 0.08). A phylogenetic tree constructed from a dataset of DNA sequence SNPs (single nucleotide polymorphisms) from 146 beetles representing three species within the genus *Pseudocotalpa* showed genetic divergence within a species is 0.24644 for *P. giulianii*, 0.18148 for *P. andrewsi*, and 0.17900 for *P. sonorica*. Within *P. giulianii* the genetic divergence in the Lava Dune (LD) population was 0.003967. The tree also showed Lava Dune individuals grouping together monophyletically on their own branch that is nested within the Big Dune beetles.

The Mount Charleston blue butterfly (Lepidoptera; Lycaenidae, *Icaricia shasta charlestonensis*) is a subspecies endemic to the Spring Mountains (Clark Co., Nevada), found primarily along alpine ridges above Lee Canyon and Kyle Canyon at elevations between 2500 and 3500 meters. *I. s. charlestonensis* is listed as endangered due to threats to its habitat, its small population size, and extremely limited larval host plant range. Sequencing of mitochondrial DNA (mtDNA haplotypes) and nuclear DNA (SNPs) from butterflies sampled at

four locations provided two genetic data sets that were used to elucidate fine-scale genetic divergence among butterfly subpopulations in upper Kyle Canyon and upper Lee Canyon. Analysis of molecular variance using eight mtDNA haplotypes did not identify any genetic structure (ΦST 0.0101) between populations. In contrast, the DNA dataset containing more than 101,000 SNP genetic markers provided evidence of substantial genetic structuring present among *I. s. charlestonensis* subpopulations within the Spring Mountains (Fst: 0.094, Nm: 6.326). A phylogenetic tree created using the SNP dataset suggests there is one monophyletic grouping of butterflies within the South Loop subpopulation, distinct from the other three locations in Lee Canyon Phylogenetic analysis, Principal Coordinate Analysis, identification of 3 or 4 distinct genetic units using the software STRUCTURE and estimates of genetic parameters reveal the underlying patterns of genetic structure. The Kyle Canyon subpopulation of butterflies, geographically isolated with no intervening habitat is genetically distinct with little evidence of mixing with the other three subpopulations in Lee Canyon. Genetic exchange among subpopulations (gene flow) appears to be greatest among the sites that are geographically proximate in Lee Canyon. Overall, this endangered subspecies exhibits fine-scale genetics structure within the Spring Mountains, a finding with important implications for future management of butterfly habitat and genetic diversity.

In this study I demonstrate how the evolutionary history of each species has been shaped by fine-scale patterns relating to ecological specialization. Resources that these specialized species have evolved to exploit include the host plants of the butterfly *Icaricia shasta charlestonensis* and limited sand accumulation utilized by *Pseudocotalpa giulianii*. The geographic structure of these resources shape the phylogeographic structure and genetic diversity of these endemics with limited distributions in southern Nevada. Population and phylogenetic

analysis of *P. giulianii* and *I.s. charlestonensis* revealed relatively low connectivity among some subpopulations over relatively short geographic distances and evidence that connectivity and genetic exchange is closely tied to the ecological distribution of resources within their respective ranges.

Acknowledgements

I would like to thank my graduate committee advisor Dr. Daniel Thompson (School of Life Sciences) for accepting me as a student, for opening his door to a new face to offer professional guidance, and for encouraging my interest in biodiversity. I thank my committee members: Dr. Donald Price (School of Life Sciences) for providing expertise in the field of evolutionary biology; Dr. Allen Gibbs (School of Life Sciences) for providing essential resources for my research project and the opportunity to develop new skills; Dr. Margo Odlum (Department of Geoscience) for providing expertise regarding geology. I also thank Dr. Richard Tillet (formerly at UNLV's Nevada Institute of Personalized Medicine) for providing hands-on bioinformatics training, data analysis and expertise on computational analyses used in this project; Dr. Sean Neiswenter (School of Life Sciences) for providing resources and molecular training; Dr. Jef Jager (School of Life Sciences) for his professional guidance and willingness to lend a helping hand; and Dr. Matthew Van Dam (California Academy of Science) for providing rare *Pseudocotalpa* specimen used in this study and sharing ecological knowledge about *Pseudocotalpa*. I also thank the Bureau of Land Management and U.S. Fish and Wildlife, for granting funding, without which this project would not be possible. I thank Corey Lange (Bureau of Land Management) for organizing field outings, conducting surveys at the sand dunes, and providing resources and beetle specimen. I also acknowledge the Graduate and Professional Student Association for granting me funding to conduct fieldwork. Many thanks to my fellow graduate students and friends for being a part of my experience at UNLV. Special thanks to my fiancée, Cameron Yee, for his never-ending support. Finally, I thank my family and parents, Eileen Tovar, and Juan Tovar for their love and faith filled guidance throughout my life. Deo gratias.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

Ecological specialization and geographic isolation are factors that can shape patterns of species divergence at evolutionary time scales (Dupuis et al., 2020). Bringing together both is imperative to understanding population genetic structure and lineage diversification. Phylogeographic studies across many taxa have examined the population genetic processes that contribute to diversification (Chan et al., 2020; Nguyen, 2019; Schierenbeck 2014). Most of these studies investigate divergence across broad and diverse geographic areas, and thus do not assess fine-scale population structure of species at small geographic scales. Further, animals with localized endemism, host specificity, and geographic isolation lack representation in these studies.

Ecologically specialized species typically have their ranges limited to the geographic distribution of resources for which they have evolved unique phenotypic traits that allow resource exploitation. Resources can be consumable (Dennis, 2004) such as diet plants, or non-consumable structural environmental elements such as shrubs used for mate location or substrates used for shelter. Two well-known resources that limit insect species are specializations on one or a few hosts, typically plants, and specialization related to soil substrate such as sand (Dennis et al., 2014). For example, many insect species have evolved morphological structures and behaviors related to burrowing which may limit the insect's distribution to sand areas with specific conditions relating to the composition, compaction, moisture level, and granule size of sand (Min et al., 2018).

If essential consumable or non-consumable resources are limited in geographic distribution or spatially discontinuous or patchy, species specialized to utilize these resources

will exhibit locally restricted population distributions, geographic isolation and/or small population sizes compared to less specialized species (Brown, 1984; Dupuis et al., 2020; Lester et al., 2007; Nolte et al., 2019). The evolutionary and population genetic consequences of isolation and small population size are a lower total genetic diversity and a potentially higher extinction risk than a larger or more connected population (Chan et al., 2020, Stockwell et al., 2003; Thompson, 2020).

In research on two restricted insect species in southern Nevada, I have examined the population genetic consequences of isolation and small population size and assessed whether fine-scale geographic restrictions in specialized habitat factors cause small-scale phylogeographic structure and genetic isolation within the already restricted ranges of endemic species. Restrictions related to ecological specialization include the host plants of the butterfly *Icaricia shasta charlestonensis* in the Spring Mountains and the limited sand accumulation at Big Dune and Lava Dune for *Pseudocotalpa giulianii*. The geographic structure of these resources may have profound implications for the phylogeographic structure, genetic diversity, conservation biology, and management of these endemics limited to southern Nevada.

In both taxa, there exist geographic and resource restrictions that have the potential to lead to divergence among localized subpopulation areas and phylogeographic structure. Big Dune and Lava Dune, the only dunes where *P. giulianii* reside, are separated by 7 kilometers of desert lacking habitable sand and a highway bisecting the dunes. I hypothesized *P. giulianii* subpopulations would show genetic divergence due to restricted gene flow between the two sand dunes. I hypothesized Big Dune, the larger sand dune where the majority of *P. giulianii* reside, would have a beetle population with more mitochondrial DNA haplotypes and higher genetic diversity due to its larger population size and longer evolutionary history than Lava Dune. If this

prediction is correct, Big Dune and Lava Dune beetles will show phylogenetic divergence and haplotypes unique to each dune. Alternatively, analyses may detect the same haplotypes in both populations and no phylogenetic divergence, suggesting that movement across desert areas without sand habitat occurs at a high enough level to maintain gene flow between the two populations.

For the butterfly *I. s. charlestonensis*, I predicted the fragmented, patchy distribution of larval host plants within the Spring Mountains would cause modest to moderate genetic divergence among subpopulations based on observations of limited and highly localized movements of adults (Austin, 1980). Under a hypothesis of fine-scale geographic isolation, I expected the South Loop butterfly subpopulation to exhibit the greatest genetic divergence from other subpopulations due to the lack of larval host plants across a 2 mile stretch of mountain slopes between Kyle Canyon and Lee Canyon. Alternatively, I predicted there would be little or no genetic divergence across the Spring Mountains if butterflies moved longer distances, more typical of other Lycaenid species (Peterson, 1996) and used small patches of larval host and nectar plants to travel across the geography of the Spring Mountains. This would suggest that Mount Charleston blue butterflies are not limited by their specialization on a small set of host plants.

Chapter 2: *Pseudocotalpa giulianii*

The order Coleoptera, commonly known as beetles, are the most species rich insect group, containing 25% of all described species on Earth, with an evolutionary history dating back to the Permian (Zhang et al., 2018). Because of their diversity and long evolutionary history, beetles are an ideal group for the study of a wide range of evolutionary questions, including those relating to fine-scale habitat structure and genetic divergence. Although there have been many studies pertaining to genetic divergence of populations within the order Coleoptera, relatively few attempts have been made to assess local population genetic divergence of populations, and none at the fine geographic scale represented in my research.

The high species richness of Coleoptera can be attributed to their low lineage extinction and long evolutionary history (Hunt et al., 2007). Plant feeding is also theorized to have led to the rapid expansion of beetles; as angiosperms diversified, some beetle clades co-diversified along with angiosperms (McKenna, 2019). Beetles exhibit high levels of diversity, displaying various morphological differences and playing important roles in terrestrial and freshwater ecosystems. Beetles are all characterized by hardened fore wings called elytra, that cover the hind winds. In most species the elytra are raised during flight, but some beetles have no wings and are flightless. Like other insects that have a head, thorax, and abdomen, although the divisions of the thorax are usually only visible on the ventral side. They have three pairs of legs that can vary in shape from hooked to thick paddles for swimming (Mullen and Durden, 2002). All beetles exhibit complete metamorphosis development. Eggs are typically laid on or in soil or plant matter. Larvae exhibit diverse morphology and usually have a hardened head and chewing mouthparts. Beetle larvae molt at least three times from instar to instar before transforming into pupae.

In 1974, Hardy described *Pseudocotalpa giulianii* from south central Nye Co, near the border of California and Death Valley National Monument, known as Big Dune. In his original description, he distinguished the genus *Pseudocotalpa* from closely related genera in Aerodina by the deeply concave clypeus and the poorly developed prothoracic post-coxal spine. *Pseudocotalpa* beetles possess wings and hooked tarsal claws, used in mating by male beetles to grasp females during copulation. *Pseudocotalpa* beetles are considered sand dune obligates – they need sand deep enough and with enough plant matter and moisture to lay eggs and develop in the larval and pupal stage. Pseudocotalpa feeds during the larvae stage and has not been observed feeding in the adult stage. The larvae are covered in hairs, likely for sensory purposes as they navigate in the sand for to feed on plant and debris. Adult fat reserves have been shown to decrease over the mating season in both sexes. They are drought tolerant, adult *Pseudocotalpa giulianii* have been observed emerging and mating after a year of effectively no precipitation.

The life cycle of *Pseudocotalpa* is typical of scarab beetles. The egg hatches as a larva, following several instar stages where the larva is increasing in size before metamorphizing from the pupal stage to an adult beetle. The exact amount of time *Pseudocotalpa* spends in the larval and pupal stages is unknown, scarab life cycles vary with climate, with temperate regions requiring longer life cycles. Life cycles from one to three years have been recorded in Rutelinae (Ritcher, 1958). However, it is thought *Pseudocotalpa* species spend most of their lives in the larval stage under the sand, consuming organic matter that is largely made of plant material (Hardy, 1976; Rust 1985). Alternatively, the majority of their lives could be spent as a pupae, where they wait for specific environmental cues before metamorphosing as an adult beetle (Ritcher 1958). These beetles do not feed in the adult stage, instead relying on fat reserves obtained in the larval stage.

The reproductive behavior of *Pseudocotalpa* is unique. Annually, the adult *P. giulianii* emerges from the sand on almost the exact date in late April. In the late afternoon, about 1 hour before dark, a small horseshoe shaped divot in the sand appears, followed by the eyes and head of the beetle (Hardy, 1976). After several minutes the adult will emerge and perch on the surface of the sand before taking flight. Typically, the beetles fly toward a creosote bush and land or hover over other individuals on the bush. If a male detects a female, he will attempt to copulate on the branches of the Creosote before they both fall onto the sand beneath the bush. If no female is present, he will fly away to another bush. Beetles can be observed copulating on bushes or dune swales across the dune for 45 to 60 minutes before burying themselves in the sand once again. This mating event will take place every evening for 30 - 40 days, after which the males use up their fat reserves first and die. Male life span was found to be 20 days, while females lived longer, an average age of 48 days. This is likely due to behavior differences, where males fly more in search of females and females conserve energy in order to lay eggs beneath the sand.

Habitat specialization

Pseudocotalpa beetles are considered sand dune obligates – they need sand deep enough to lay eggs and develop in the larval and pupal stage. They are drought tolerant, adult *P. giulianii* have been observed emerging and mating after a year of effectively no precipitation (Tovar, observation, 2022). Habitat specialists use particular landscape features and vegetation in their environment, this can limit populations to spatially isolated areas of useable habitat (Chan et al., 2020). Increased habitat specialization has generally shown to increase the risk of extinction (Tscharntke et al., 2002). This is especially true for insects when compared to other animal taxa (Dunn, 2005). In a study on 464 carabid beetle species, habitat specialization was shown to be the most significant predictor for extinction risk (Nolte, 2019). This is linked to increases in

habitat disturbance typically associated with land-use change like urban development and agriculture (Nolte, 2019). Small distribution range size and large body size were also significantly associated with risk of extinction. However, for carabid beetles that lived in forest habitat, high trophic level rather than habitat specialization was shown to be the most significant predictor of extinction risk. Swedish longhorn beetles had two best predictors for extinction risk: population decline and small fragmentated populations. However, habitat specialization and body size were also found to be contributing factors (Jeppson and Forslund, 2014).

The generalist feeding nature of *P. giulianii* larvae and lack of host specificity suggests that it will be somewhat adaptable to changing environments (Tscharntke et al., 2002). However, as with all animals, there are certain habitat criteria that are required for this beetle to persist. The strength of these relationships and the extent to which the habitat can be altered and remain viable remains yet to be explored.

Pseudocotalpa beetles have the ability to fly, suggesting they might have higher movement capability than the flightless carabid beetles (Nolte et al., 2019). However, there are several limits to their dispersal capabilities. The highway can limit beetle dispersal (Muñoz et al., 2015) and pose as a recent barrier to gene flow between the populations (Keller and Largiader, 2003). In addition, *Pseudocotalpa* beetles do not feed in the adult stage, their limited energy store last for 5 to 7 weeks, after which they die. Their flight pattern is indirect, generally they fly from bush to bush searching for a mate. *P. sonorica* and *P. andrewsi* spend less time hovering over bushes than *P. giulianii*, where *P. andrewsi* appears to have the fastest flight pattern. *P. giulianii* has only ever been observed flying short distances while attempting to copulate with female beetles (Hardy, 1976). This short adult stage, combined with their flight pattern likely limits their dispersal capability. However, the beetles can theoretically disperse to nearby dunes

by flight, but it is unlikely that it is a regular occurrence. The 7 km distance between the two *P. giulianii* populations and the highway in between likely pose as barriers to dispersal. It's more likely that this genus dispersed to different dunes from warm wind-up currents or a storm system (Onstad, 1999; Wiktelius, 1981).

P. giulianii could disperse to other dunes nearby like Ash Meadows, Saline Dune, Eureka Dune, and Death Valley dunes. These dunes are much closer than the Algodones Dunes, where the other two *Pseudocotalpa* species are found. However, *P. giulianii* has not been found on these regularly visited dunes; these beetles can be easily found dead on top of the sand for several months after the end of mating season. While *Pseudocotalpa* could have existed on nearby dunes, colonization events are not usually successful (Sol, 2008). In addition, *Pseudocotalpa* has narrow soil requirements, which restrict it to sandy routes left by receding lakes and rivers of the Pleistocene hydrographic system (Pavlik, 1985).

The Algodones Dunes where *P. andrewsi* and *P. sonorica* populations reside are around 400 km from Big Dune. The Algodones Dunes is bisected by a highway, which divides the wilderness area from the recreation area. In the northern wilderness area, *P. sonorica* populations have been reported whereas *P. andrewsi* populations have historically been found in the recreation area. The recreation area has less vegetation compared to the wilderness area due to extensive ORV use. Vegetation is thought to be required for Pseudocotalpa survival, as the larvae feed on detritus from plant material. Using light-bucket traps on either side of the highway, *P. sonorica* populations were found in far fewer numbers in the recreation compared to the wilderness area (Van Dam and Van Dam, 2008). However, as population count data does not exist prior to recreational use of the dune, it could be argued that the population had previously been localized to the wilderness area, or that two seasons of population counts are not sufficient

to draw conclusions. Considering previous reports of habitat degradation due to ORV use (Shultz 1988, Luckenbach, 1983; Van Dam and Van Dam, 2008) and *Pseudocotalpa's* reliance on vegetation as a food source, it is logical to conclude ORV use similarly led to population decline. It is also feasible genetic effects from fragmentation exist for *Pseudocotalpa andrewsi* and *sonorica* populations, given the extensive habitat destruction caused by off-road vehicles (ORV). Genetic analysis might reveal a recent population bottleneck in the *P. sonorica* or *P. andrewsi* populations that can be linked to habitat destruction.

Materials and methods

Taxon sampling

I obtained 194 *Pseudocotalpa* specimens collected from the Amargosa Valley, Nevada and Algodones Dune, California. Of these specimens, 140 were *P. giulianii*, 32 *P. andrewsi* and 22 *P. sonorica*. Of the 140 *P. giulianii* specimen, 91 were from Big Dune and 49 were from Lava Dune.

Mitochondrial DNA laboratory methods

I obtained 594 – 680 base pairs (bp) of cytochrome oxidase I mitochondrial DNA sequence data from the 194 *Pseudocotalpa* beetle specimens. Genomic DNA was extracted from 198 samples using the Qiagen DNeasy Blood and Tissue Kit following the Supplementary Protocol for Insects (Qiagen, Valencia, CA, USA), 4 samples did not yield DNA despite re-extraction and re-amplification attempts. DNA was amplified and sequenced at the cytochrome c oxidase subunit I (COI) region of the mitochondrial DNA, using the universal primers LCO-1490 and HCO-2198 (Folmer et al., 1994). I performed re-amplifications from the DNA re-extractions using primers LCO-1490 and HCO-2198 as well as additional COI primers Nora1736 and Ron (Ugelvig et al.,

2011). I amplified and sequenced the nuclear internal transcribed spacer region 1 (ITS1; Wilson et al., 2013). Additional mtDNA sequences available from Genbank were included as outgroups in phylogenetic trees. The trimmed CO1 alignment compromised 594 base pairs from 161 individuals; the sequences of the *P. sonorica* has slightly longer sequences (639 bp). To examine the mitochondrial DNA sequence diversity in the two populations, haplotype networks were constructed using the software PopArt 1.7 (Leigh and Bryant 2015). DnaSP was used to determine genetic parameters including number of haplotypes, segregating sites (S), nucleotide diversity (π), and theta (Θ), and to conduct Tajima's D, Fu's F, Strobeck's S, and mismatch distribution tests of neutrality. Phylogenetic trees were constructed using maximum-likelihood (MEGAX 10.18, Figure 2) and Bayesian (Phylogeny.fr; Figure 3) methods. Following model-testing with maximum-likelihood methods, I selected the best model fit for each tree.

Genotyping-by-sequencing using nuclear DNA

I chose 165 beetle samples for genotyping using GBS technology utilizing the restriction enzyme Bsp 126I. A de novo assembly was used as the reference genome for this project. Sample were sequenced using an Illumina HiSeq X instrument, a total of 2 x 308,886,002 reads was generated with an average of 2 x 1,872,036 reads per sample. Each individual sequence read was trimmed of low-quality regions and aligned to the reference genome using GNAP (Wu and Nacu 2010). Only reads with a single unique alignment were used for analyses. A SNP was called as homozygous in a diploid sample if at least 5 reads supported the major common allele at that site and at least 90% of all aligned reads shared the same nucleotide at that site. A SNP was called as heterozygous in a diploid sample if at least 2 reads supported each of a minimum of 2 different alleles and each of the 2 allele types separately made up more than 20% of the reads aligning to that site and when the sum of the reads supporting those 2 alleles equaled 5 and

made up at least 90% of all reads covering the site. The sites were further filtered to obtain a set of 152,159 SNPs. 19 of the original samples were removed during the filtering process due to a high missing rate including all three *Pseudocotalpa* species and outgroup species *Paracotalpa punticolis, Paracotalpa granicolis and Aphodis sp.*.

These SNPs were further filtered by defining an acceptable minimum call rate (MCR) of $\geq$ 50% per SNP. This dataset, MCR50P, contained 99,915 SNPs from 146 samples (Table 2). The MCR50IN SNPs were implemented in GenoDive v2,0b27 (Meirmans and Van Tienderen 2004) to calculate descriptive population genetic statistics and tested for pairwise population differentiation. The MCR50P SNPs were used to construct a phylogenetic tree (Figure 11). To construct the phylogenetic tree from the MCR50P SNP dataset, pairwise distances were estimated between MCR50 individuals using an unbiased model of substitution frequencies. Neighbor-Joining algorithm was used to construct a phylogenetic tree using distance estimates (Saitou and Nei, 1987) and performed in the NJS module of the APE (Paradis et al., 2004) R package.

Results

Phylogenetic analysis of mitochondrial DNA

Mitochondrial DNA sequences of *Pseudocotalpa* and related outgroups were used to create maximum likelihood and Bayesian phylogenetic trees. Of the 140 *P. giulianii* sequences, 91 were from Big Dune and 49 were from Lava Dune. Maximum likelihood phylogenetic trees of the COI haplotypes were constructed with two sets of congeners and outgroup species from Genbank. The first utilized 594 bp sequence from all three *Pseudocotalpa* species, the *P. sonorica* sequences were slightly longer (639 bp) from the Amargosa Valley and Algodones

Dune: 140 *P. giulianii,* 15 *P. andrewsi* and 6 *P. sonorica*. The second group of species contained 17 P. andrewsi and 16 P. sonorica with 680 bp of sequence data. Both utilized COI sequences from *P. giulianii, Cotalpa lanigera, Paracotalpa ursina, Chrysina aurilisternum, Chrysina bruyeai, Chrysina aurigans, Chrysina limbate* and *Chrysina luteomarginata.*

This tree (Figure 2) is rooted at the *Chrysina sp.* and *Paracotalpa punticolis* node. Maximum likelihood phylogenetic trees of the COI haplotypes revealed a phylogeny with well supported species groupings for *P. giulianii* (100%) across all 9 haplotypes. There is strong support at the basal nodes of this tree (99 – 100%). Four basal nodes show high support for phylogenetic groupings. The outgroup species *Chrysina aurigans, Chrysina bruyeai, Paracotalpa ursina, Chrysina limbata,* and *Chrysina aurilisternum* have 100% bootstrap support. All three *Pseudocotalpa* species form a grouping with 100% bootstrap support at their basal node, excluding three sonorica individuals. Two *P. sonorica* and all *P. giulianii* haplotypes form a grouping with 99% bootstrap support. All *P. giulianii* haplotypes are grouped together with 100% bootstrap support, forming a monophyletic grouping. *P. andrewsi* individuals form a monophyletic grouping. However, there is low bootstrap support for *P. andrewsi* monophyletic grouping at the basal node (48%). Shallow groupings within *P. andrewsi* individuals range from 20% to 100%. *P. sonorica* was grouped in two locations on the tree, one grouping branching near *P. giulianii* and *P. andrewsi* with 99% bootstrap support and one grouping near outgroup species with 70% bootstrap support. *P. sonorica* groupings show high support (99%) and low support (70%).

The Bayesian tree used the same COI sequences and showed higher bootstrap support across the nodes along with similar groupings. Bayesian analysis shows a similar phylogenetic structure, with the same outgroup species grouped together at the most ancestral node (Fig. 2).

P. giulianii is forms a monophyletic grouping (100%). *P. sonorica* and *P. andrewsi* group with *P. giulianii* (100%), except for three P. *sonorica* that remain outside of that grouping and form a grouping with outgroup species *Cotalpa lanigera*, *Chrysina peruviana* and *Chrysina aurilisternum* (100%). Chrysina aurigans is the most distant outgroup on this tree.

Haplotype network of mitochondrial DNA

The 140 *P. giulianii* for which I have mtDNA, collected from two sand dunes in the Amargosa Valley (see Methods mtDNA), yielded 5 unique haplotypes. A minimum spanning network for those *P. giulianii* haplotypes illustrates the relationship between Big Dune and Lava Dune, Nevada (Fig. 3). The most common haplotype BD10_2021 is shared by both Big Dune and Lava Dune populations. The second most common haplotype LD10_2020, is found only in the Lava Dune population. The three other haplotypes are less common and only found in the Big Dune population. The 5 unique haplotypes were similar to each other, differing by 1 to 4 base pair changes. The maximum number of nucleotide differences between *P. giulianii* haplotypes was 3.

The haplotype diversity in both locations was quite low with only 5 haplotypes, despite the high number of *P. giulianii* beetles sequenced. Of the 140 beetles analyzed, 49 were from Lava Dune and 91 from Big Dune. In the Big Dune population there are 4 haplotypes, whereas the Lava Dune only has 2 haplotypes. Of the Lava Dune individuals, 85.5% are the LD10_2020 haplotype (42/49) and 14.3% are the BD10_2021 haplotype (7/49) found on both dunes. Of the Big Dune population 81.1% are the shared BD10_2021 haplotype (73/90), 14.4% are the BD15_2020 haplotype (13/90), 3.3% BD2_2021 type, and 1.1% are the BD3-21 type (1/90).

Genetic diversity of mitochondrial DNA

This genetic diversity parameters shows population structure in the *P. giulianii* species. The mean FST value, representing genetic variance across the populations is 0.76650 (Table 4). Gene flow across Big Dune and Lava Dune populations is represented by the number of migrants per generation Nm = 0.83 (Table 4). The same pooled populations of *P. giulianii* was also found to have 5 unique segregating sites (haplotypes) in DnaSP (Table 3). In addition to 5 segregating sites, in both populations there was a haplotype diversity of 0.57708, and nucleotide diversity of 0.00185.

From the 140 sequences in the sample of both Big Dune and Lava Dune populations, Tajima's D was 0.36892, a value greater than zero. This implies that there is a deficiency of rare segregating sites compared to nucleotide diversity, suggestive of a recent population bottleneck (Tajima 1989). However, this statistic was found to not be significant ($p > 0.1$). As with many phylogenetic studies, I am assuming there is no selection of existing genetic variants (Allendorf et al. 2010), noting that natural selection can also lead to deviation of Tajima's D from zero.

Other measures of neutral equilibrium deviation from a constant population size are Fu's (Fu's F = -1.20086, $p > 0.10$), Strobeck's S (Str S = 0.466), and a unimodal mismatch distribution (pairwise differences) were calculated with simulations in DnaSP. Strobeck's S statistic is the probability of having equal or fewer haplotypes than observed. A negative value of F suggests for an excess number of alleles, while a positive value suggests a deficiency of alleles. One would expect Fu's F to become more negative as Strobeck's S approaches 1. Fu's F result point toward a demographic interpretation of an expanding population, while Tajima's D point toward a recent population bottleneck. (Ramírez-Soriano et al. 2008). However, the p value for Tajima's D and Fu's F report these vales as not significant.

Nuclear data phylogenetic tree

The maximum neighbor joining phylogenetic tree constructed from the nuclear DNA MCR50P dataset clearly shows a monophyletic grouping for all three *Pseudocotalpa* species (Figure 5). *P. sonorica* is a sister taxon of *P. giulianii* and *P. andrewsi* is the most ancestral species, being slightly closer to the outgroup species *Cotalpa flavida.* In addition, the *P. giulianii* Lava Dune specimens all branch from the Big Dune clade of samples, suggesting that the Lava Dune population was colonized by the Big Dune population. The genetic divergence within a species is 0.24644 for *P. giulianii*, 0.18148 for *P. andrewsi*, and 0.17900 for *P. sonorica*. Within *P. giulianii* the genetic divergence in the Lava Dune population was 0.003967.

Discussion

Big Dune is a semi-stable small dune, estimated to have existed at least since the early Holocene, around 11,700 years ago (Pavlik, 1989). The presence of an endemic insect reflects its stability and age, unlike younger more rapidly shifting sand dunes that are characterized by low numbers of plant and insect taxa (Pavlik, 1989; Andrews et al., 1979). Big Dune is characterized by one of the Mojave desert's most widespread and common perennial plants, the Creosote bush (*Larrea tridentata*). Other plants on the dune include the sandpaper plant (*Petalonyx thurberi*), prickly poppy (*Argemone corybosa*) and astragalus (*Astragalus lentiginosus var. variabilis*). These plants all contribute to the debris underneath the sand, the food source of *P. giulianii* larvae. Because vegetation provides the debris that the beetles feed on, it is an essential resource for their survival on the dune. Vegetation also assists with sand accumulation, dune formation, and plays a role in the prevention of the loss of surface sands by wind and water (Van Dam 2008, Wigg et al., 1995). In addition, vegetation also provides structural elements used by *P. giulianii* for mate location (Dennis, 2004; Hardy, 1976). These essential resources all serve to restrict the

geographic range of *P. giulianii*, a specialized sand dune obligate species. In my research on two restricted species, I examined population genetic consequences of isolation and small population size and determined if small-scale geographic restrictions in specialized habitat cause small-scale geographic structure and genetic isolation within the already restricted ranges of insects. The results from the mtDNA and SNP analysis indicate that there is evidence of genetic divergence and population differentiation due to limitations in the geographic distribution of essential resources. This population genetic analysis of the mtDNA and nuclear DNA sequences are the first analysis of the genetic diversity and population structure of *Pseudocotalpa giulianii* beetles in the Amargosa Valley.

Phylogenetic analysis

Bayesian and maximum likelihood analyses trees (Figure 2, Figure 3) are rooted with the outgroup species *Chrysina luteomarginata, Chrysina bruyeai, Chrysina aurigans, Chrysina limbata* and *Paracotalpa ursina* as they are considered more ancestral species (Jameson 1997, Zhang et al., 2018). *Pseudocotalpa* are specialized sand dune species, in the genus there are only 3 species, and they are restricted to sand dune habitats. In contrast the other genera and species – *Cotalpa lanigera, Paracotalpa ursina*, and the *Chrysina sp.* are not restricted to dune habitats. Evolutionarily there is persistence in *P. sonorica* in an ancient ancestral lineage. That is suggesting *P. sonorica* is so deeply divergent from the other *Pseudocotalpa* that it is sitting outside of the genus *Pseudocotalpa*. This is evidence that *P. sonorica* is the species that diverged earliest of the three *Pseudocotalpa* species. However, the bootstrap support is low (36%) between P. sonorica and closely branching outgroup species (Figure 2). Bayesian analysis has much higher support between *P. sonorica* and the same outgroups (100%, Figure 3). The hypothesis of *P. sonorica's* deep divergence assumes that the mtDNA tree is robust however,

with longer mtDNA sequence reads I might expect to see a monophyletic grouping of P. sonorica. My larger nuclear SNP dataset proved that to be the case. The remaining two *P. sonorica* individuals are grouped with *P. andrewsi* and *P. giulianii* with high support on both trees (99 – 100%, Figure 2, Figure 3), supporting relatedness within the genus. COI data, only includes ~600 base pairs. The deeper branches are 100 percent, so I know the tree is well structured (Figure 2). When there is high level of divergence, for example with outgroup species like *Chrysina,* the tree building method cannot resolve the deeper divergences. The SNP data has far more DNA and I was able to resolve the lineages, including the bifurcating location of *P. sonorica* sequences.

Incomplete lineage sorting (ILS) is a process by which ancestral polymorphisms can persist through species divergences for several million years. A popular example would be chimpanzee DNA being present in human DNA, where over a long timescale there was no loss by genetic drive due to some individuals in a large effective population size retaining chimpanzee DNA (Hobolth et al., 2011). In Figure 2, *P. sonorica* persists in two ancient ancestral lineages, *Chrysina* and *Cotalpa*. At the time *P. sonorica* speciated, it was present in two ancestral lineages that gave rise to *P. sonorica*, thus there is sharing of *P. sonorica* genes with genes still present in other species. *P. sonorica* clusters with *Chrysina* and *Cotalpa,* implying that a *Pseudocotalpa* ancestor split from one of these two genera. If this is due to ILS and the retention of an ancestral polymorphism, this implies that the mtDNA in *P. sonorica* was present before the origin of these three genera. However, genetic drift can lead to the loss of polymorphisms over time and in fact, is highly likely in small populations. In this scenarios ILS would require a large population over a long timescale to prevent genetic loss due to drift. This scenario seems unlikely, since *Pseudocotalpa* species are geographically restricted to sand dunes

and have been subjected to habitat destruction, their effective population size has likely been less than what is required to prevent drift (Franklin, 1980).

Alternatively, hybridization between *P. sonorica, Chrysina* or *Cotalpa* species in the past could have caused the introduction of a mtDNA lineage. Introgression, a process where genetic information is transferred from one species to another as a result of hybridization, can occur if the new mtDNA increased the fitness of the individuals that inherited the mtDNA. About 10% of animal species hybridize (Mallet, 2005), and interspecific hybridization can be a source of genetic variation for these species. However, this low frequency hybrid originated mtDNA would also likely be lost by drift. It is possible that there is more than one species in my specimen that have *P. sonorica* like morphology that show genetically divergent mtDNA.

While incomplete lineage sorting and hybridization were viable explanations for unresolved lineages, the maximum neighbor joining phylogenetic tree made using SNP data (Figure 5) clearly shows monophyletic groupings for all three *Pseudocotalpa* species. *P. sonorica* is closest to *P. giulianii* and *P. andrewsi* is the more ancestral species, in contrast to the mtDNA tree that depicts *P. sonorica* as ancestral. In addition, the *P. giulianii* Lava Dune population monophyletically grouped and is nested within the Big Dune clade, suggesting that the Lava Dune population was colonized by the Big Dune population.

The mitochondrial DNA data supports the hypothesis that there is genetic differentiation between the two dunes. The mean FST value among P. giulianii populations was 0.76650 (Table 4). This genetic diversity parameters reveals population structure in the *P. giulianii* species. Gene flow across Big Dune and Lava Dune populations is represented by the number of migrants per generation Nm = 0.83 (Table 4). These two parameters suggest there is less than one migrant per generation and significant levels of genetic differentiation between Big Dune and Lava Dune.

On the SNP tree (Figure 5) genetic divergence within each species was similar, *with P. giulianii* having the greatest divergence (*P. giulianii*: 0.24644, *P. andrewsi*: 0.18148, and *P. sonorica*: 0.17900). Within *P. giulianii*, all the Lava Dune individuals group together monophyletically on a branch that is nested within the Big Dune individuals. The genetic divergence within Lava Dune population was 0.003967. Because there are multiple species on the SNP tree, this value represents a significant amount of divergence within the Lava Dune population. Comparatively, this Lava Dune grouping had genetic divergence that was at least an order of magnitude greater than most other branches within the species. This provides evidence to support the low levels of gene flow and isolation of the dunes at a fine-scale.

I observed 9 individuals branching individually on their own branch, 3 from Big Dune had high levels of genetic divergence (BD3_2020: 0.002804, BD12_2021: 0.005846, BD22_2021: 0.001303). A potential theory for this divergence is the presence of several cohorts present among our sampled individuals. This could occur if *P. giulianii* utilized facultative diapause, where their development is arrested each generation due to environmental cues (Schebeck, 2017). If this were the case, *P. giulianii* individuals would be in the sand for several years, first as an egg and larvae, then as a diapausing prepupae, before pupating and emerging to mate as an adult during the spring flight season. One might expect all *P. giulianii* to have the same environmental cues and therefore all emerge during the same season. However, the sand provides an environmental gradient from top to bottom, where each larva may experience a different temperature or photoperiod depending on what depth the larvae is present. Alternatively, the larvae are seeking the same environmental conditions, such as moisture level in the sand, where photoperiods and temperature are equal across the dune, thus syncing the larvae to the same environmental cues and pupation time. Experiments with larvae exposed to

different light and temperature conditions would illuminate the influence of diapause cues and its effect on the genetic diversity of *Pseudocotalpa*.

Haplotype analysis of mitochondrial DNA

I hypothesized *P. giulianii* subpopulations would show genetic divergence due to restricted gene flow between the two sand dunes. I hypothesized Big Dune, the larger sand dune where the majority of *P. giulianii* reside, would have a beetle population with more haplotypes and higher genetic diversity due to its larger population size and longer evolutionary history than Lava Dune. The two *P. giulianii* subpopulations are separated by less than 7 km, yet the phylogeny and haplotype data show fine-scale genetic differentiation. My mtDNA suggests that isolation between the two populations does exist, shown by the haplotype that is only present in the Lava Dune population (Figure 4). The unshared haplotype is the most abundant in the Lava Dune population, implying that there is not much gene flow between populations. If there was high levels gene flow between populations, I would expect to see all or most haplotypes shared by both Big Dune and Lava Dune.

The haplotype network (Figure 4) was created using 49 beetles collected from Lava Dune and 90 from Big Dune (total = 139). While the number of individuals is sufficient to compile the haplotype diversity of the population, the haplotype diversity is shown to be relatively low. This haplotype network has only 5 haplotypes, 2 present in Lava Dune and 4 in Big Dune, with one shared haplotype. The network shows a low diversity system, with the smaller Lava Dune population having the lowest diversity of the two populations. While the number of individuals was sufficient to compile the haplotype diversity of the population, it is likely that the length of the sequences (~594 bp) contributed to the low number of haplotypes.

In the network, I see the most abundant haplotype in Lava Dune, LD 10_2020, is not present in the 91 individuals sampled from the Big Dune population. This implies that there is no gene flow or a low level of gene flow between the two populations, which is supported low Nm value (Nm = 0.08). Another thing to note is how the most abundant haplotype, BD10_2021, is the source of the other 4 haplotypes. This implies that there was a single colonization event of the entire dune area at Big Dune. We can logically assess that the Lava Dune population has been colonized by the source, Big Dune population. From the most abundant shared haplotype found at both dunes, a new derived haplotype LD10_2020 emerges.

While this seems to be what the haplotype network is showing, the colonization could have occurred in the opposite direction. Where the initial colonization of the area occurred at Lava Dune. In this case, the smaller Lava Dune population would have colonized the Big Dune population. This would be consistent with the geology of the dune area, as Lathrop Wells cinder cone has been present for ~77,000 years (Heizler et al. 1999), much longer than the estimated 11,000-year-old Big Dune (Pavlik, 1989). Because Big Dune is a larger habitat, the beetles would be able to expand their population size and consequently, their genetic diversity. However, with a long evolutionary history we would expect to see more genetic diversity (haplotypes) present at Lava Dune, which is not reflected in the data. We must keep in mind that the habitat size at both dunes is extremely restricted, even more so at Lava Dune. This fine-scale geographic restriction would serve to restrict the population size, and therefore diversity of the populations.

It could be that Lava Dune and Big Dune have experienced small scale extinctions at each dune. For example, Lava Dune could have been colonized first with a small founding colonization, followed by colonization of Big Dune by a Lava Dune beetle. The small population

at Lava Dune could have gone extinct but the Big Dune population persisted, and a later colonization event could have repopulated Lava Dune. This appears to be the most likely scenario, as it is consistent with the geography of the region and is reflected in the haplotype network. I would expect to see a shared haplotype present at both dunes, and less diversity at Lava Dune – the pattern we see in my haplotype network (Figure 4). Alternatively, if the populations were genetically similar we would expect to see high levels of gene flow and haplotypes shared across both dunes. Parts of my data that the alternative are the shared BD10 haplotype. Therefore, future research needs to assess the amount of divergence on the phylogenetic tree (Figure 5) between Lava Dune and Big Dune individuals in order to estimate genetic divergence between the Big Dune and Lava Dune populations.

What I can definitively say about the shape of this network is that there was a colonization event that happened at some time in the past followed by isolation at Lava Dune. There is a relatively high frequency of the LD10_2020 haplotype that is not present in the Big Dune population. This suggests that Lava Dune has experienced some level of isolation from Big Dune. However, there are only two haplotypes found at Lava Dune, suggesting that the population has not experienced its own expansion of diversity. If the Lava Dune population had been isolated for a long period of time you would expect to see more haplotypes exclusively found in the Lava Dune population. Alternatively, it could be that the population is small enough that the genetic diversity has remained low. Analyzing my SNP data further will provide insight into the genetic divergence and diversity at both dunes.

Future analyses with the SNP data could use STRUCTURE and principal component analysis to determine the confidence in assigning *P. giulianii* individuals to separate populations. For example, detection of significant genetic structure in the SNP data that coincides with the

separate dunes' populations would provide robust evidence of low gene flow and geographic isolation between the dunes. In addition, genetic parameters using the nuclear data could be analyzed to measure the levels of divergence, gene flow, and heterozygosity within and among *Pseudocotalpa* populations. Another interesting study would be to measure phenotypic differences of individuals. This could reveal phenotypic divergence within and among populations that may support genetic divergence between populations.

Threats, stressors and conservation applications

Sand dunes are inhabited by diverse and endemic biota that can be designated as biodiversity hotspots or nature reserves. Desert sand dunes are small ecosystems that usually do not support endemic vertebrates but are large enough to support endemic insects (Dunn, 2005). Because insects do not receive the same amount of funding and attention as vertebrate species, their habitats can be left vulnerable to anthropogenic disturbance (Van Dam, 2008). The Antioch sand dunes in California have eight endemic insect species, three are extinct and the other five are endangered or threatened (Dunn, 2005). Big Dune has eight Coleoptera species petitioned for listing under the U.S. Endangered Species Act of 1973 (Hardy, 1976), one of those being *Pseudocotalpa giulianii* (Coleoptera; Scarabaeidae).

Off-roading vehicles (ORV) can destroy vegetation and reactivate sand dune movement, an effect that can destabilize a dune system (Luckenbach 1983; Van Dam 2008). Past studies have shown marked declines in vegetation, arthropods, lizards, and mammals in areas with ORV use compared to areas with restricted ORV used (Luckenbach 1983; Van Dam 2008). Giuliani's sand dune beetle's main habitat at Big Dune is roughly 1 km^2, the only other habitat where this beetle is Lava Dune, is even smaller, around 0.8 km^2. Because *P. giulianii's* habitat is extremely restricted, ORVs and the subsequent habitat degradation are the greatest threat to their species.

Evolutionarily significantly units (ESUs) have been used to distinguish a population for conservation management that is distinctive genetically and ecologically (Funk, 2012). An ESU is a population unit within a species that helps guide conservation efforts. When designating conservation units, habitat type, morphology, life history, and varying allele frequencies should be taken into consideration. Problems can arise when conservation practices rely too heavily on incomplete molecular data. Over splitting of species can occur like with the little brown bat, where mtDNA showed genetic differentiation in two geographically distinct populations. Further analysis using microsatellite data showed little genetic differentiation; this along with little morphological differentiation showed lack of evidence for the subspecies designation (Lausen 2008).

Will the level of structuring be high enough to designate separate ESUs? I know from the mtDNA that there is one shared haplotype in both populations and one unshared haplotype in the smaller Lava Dune population. When I can see genetic differentiation with such a small amount of mtDNA, it is likely that I will see more haplotypes that are not shared between populations with a larger dataset. I do see one shared haplotype, BD10_2021, suggesting some low level of gene flow exists between the two populations. Since both Big Dune and Lava Dune habitat have uniformly distributed vegetation and terrain, I would expect to see no genetic structuring within populations. However, there is some population structuring among populations due to fine-scale geographic separation and limited gene flow. There are only two populations of *P. giulianii*, my SNP analysis (Figure 5) shows that *P. giulianii* is monophyletic, providing evidence that the two are subpopulations not genetically distinct enough to warrant separate ESUs. It is unlikely that the genetic differentiation will be high enough to warrant separate ESUs. The larger SNP dataset, when analyzed in future work, will inform the level of structure present for *P. giulianii*

populations. Additionally, having separate ESUs would likely not be useful as they are so close in geographic distance and can be managed in a similar manner.

It would be informative to compare the genetic diversity and structure of the large *P. giulianii* population at Big Dune to *P. andrewsi*, a species listed a critically imperiled in the state of California (CDFW, 2021). This will allow managers to reevaluate or confirm protection statuses for these species. For example, if the Big Dune populations level of genetic diversity is about equal to or less than *P. andrewsi* population, managers could make a case for listing status or conservation efforts. Alternatively, *P. giulianii* could have high or average levels of diversity, which would leave conservationists less concerned. However, habitat destruction and small habitat size remain a concern regarding *P. giulianii*. In 2022, beetle population counts were much lower than counts from the previous two seasons. This is likely due to the recent drought experienced in the Mojave Desert. Managers could still opt for a preventative conservation approach. I would suggest closing ORV access to the vegetated area on the northeast area at Big Dune, while keeping the main dune area open to ORV use. Currently this area is designated as protected by the Bureau of Land Management (BLM), however ORV users have created paths to drive through the habitat that continue to be used. Public education and outreach to ORV users about the unique beetle species *Pseudocotalpa giulianii* and its habitat could reduce the use of ORVs in critical habitat. In conjunction with increased monitoring and enforcement of protected habitat and/or fences that cannot be easily driven over by ORVs should be installed to protect beetle habitat.

Chapter 3: Mount Charleston Blue Butterfly

The Spring Mountains of southern Nevada are geographically isolated from other mountain ranges by ~80 miles of low elevation desert. It is the highest elevation range in the Mojave Desert and is the southern end of north-south mountain ranges of the Great Basin (Austin, 1980). The Mount Charleston blue butterfly, *Icaricia shasta charlestonensis* (Family: Lycaenidae), endemic to the Spring Mountains, has an extremely limited range of about 1.44 hectares (FWS 2021). The Mount Charleston blue butterfly was listed as endangered in 2013 and critical habitat was designated in the summer of 2015. Habitat requirements include host plants to complete its life cycle and nectar plants for feeding in the adult stage. Threats to this species include destruction of habitat from forest succession and changes in natural fire regime, grass or shrub overgrowth and non-native plants, development or fuels reduction projects, disturbances by recreation activity and feral horse activity.

The Mount Charleston blue butterflies are difficult to observe because of their low abundance at low elevation sites and occupied butterfly habitat lie in difficult to access and remote terrain. George Austin described the subspecies and showed that there were annual and seasonal fluctuating population numbers in his monitoring surveys (1980). Prior to more recent population surveys, *I. s. charlestonensis* was believed to only occupy Lee Canyon ski area. These surveys led to the discovery of an abundant population of *I. s. charlestonensis* butterflies at a high elevation along the South Loop Trail, with observations of 50 to 100 butterflies in 2010 and 2012 (Sever, 2011; Thompson, 2015). Butterflies also had a population increase in 2015 and 2016 and a stable population in 2019 and 2021 along the Bonanza Trail (Thompson, 2022). While population surveys are useful for estimating population numbers, they do not give insight into the structure of populations necessary for understanding population dynamics.

This project summarizes genetic sampling and assesses genetic diversity, population structure, and differentiation among geographically isolated habitat of the subspecies range. In a similar study, Crawford (2011) used microsatellites to observe fine-scale genetic structure for another endangered butterfly the Mormon metalmark butterfly (*Apodemia mormo*). Many small subpopulations of the metalmark experienced low levels of gene flow or high genetic drift at a scale of less than 20 km. Using next-generation sequencing, hundreds of thousands of SNP markers were detected in the MCBB genome. Information regarding the population's status will also be of value for conservation and management efforts.

Materials and methods

Taxon sampling

Due to the protected status of *I. s. charlestonensis* and the failure of non-lethal methods to collect samples for genomic analysis, collection was limited to opportunistic sampling of senescing males with worn wings under the approved permit (TE63440B-0, 2015 and TE63440B-1 2019 -2024). A total of 25 *I. s. charlestonensis* were collected and preserved in EtOH for sequencing (Table 8). In addition, the genetic samples include 15 other butterflies collected from the Spring Mountains and museum collections: a male *I. s. minnehaha* sampled from Wyoming, four *Icaricia icarioides austinorum,* two *Icaricia acmon,* five *Euphilotes ancilla purpura* and three *Euphilotes ancilla cryptica.*

Mitochondrial DNA laboratory methods

The 25 *I. s. charlestonensis* samples were extracted for mtDNA and sequenced at the USDA Forest Service National Genomics Center for Wildlife and Fish Conservation in Missoula Montana. A portion of DNA from each sample was sent to a commercial laboratory, Data2Bio,

for next-generation genotyping-by-sequencing (GBS). The mtDNA was extracted using the Qiagen DNeasy Blood and Tissue Kit following the Supplementary Protocol for Insects (Qiagen, Valencia, CA, USA). The mitochondrial COI was amplified and sequenced using primers and methods described by Ugelvig et at (2011). I performed re-amplifications from the DNA re-extractions using primers LCO-1490 and HCO-2198 as well as additional COI primers Nora1736 and Ron (Ugelvig et al., 2011). I amplified and sequenced the nuclear internal transcribed spacer region 1 (ITS1; Wilson et al., 2013). Additional mtDNA sequences available from Genbank were included as outgroups in phylogenetic trees. From three fragments in the COI region I obtained 1,224 bp of sequence data for every *I. s. charlestonensis* sample. One additional sample was added in the mtDNA analysis for a total sample size of 26 (Thompson 2017). These sequences were also aligned and compared to outgroup samples previously mentioned.

Mitochondrial DNA analyses

The sequences were aligned in MEGAX 10.18 using ClustalW (Sievers et al., 2011) and the population parameters were estimated in MEGAX and DnaSP (6.12; Rozas et al., 2017). DnaSP was used to determine genetic parameters including number of haplotypes, segregating sites (S), nucleotide diversity (π), and theta (Θ). I also used DnaSP to conduct Tajima's D, Fu's F, Strobeck's S, and mismatch distribution tests of neutrality to detect demographic events such as population bottleneck or population expansion (Tajima 1989; Fu 1997, Ramírez-Soriano et al., 2008).

I constructed a haplotype network for COI using the median joining and TCS algorithm in POPART 1.7 (Leigh and Bryant 2015). I used the Analysis of Molecular Variance (AMOVA GenAlEx 6.5; Peakall and Amouse 2006, 2012) to estimate subpopulation differences in COI haplotype frequencies with 3 subpopulations (Bonanza Trail, South Loop Trail, and Lee Canyon

ski area) with 9,999 permutations. The ΦST parameter from AMOVA is the haplotype equivalent of Fst that is the proportion of genetic diversity attributable to the differences among subpopulations (Holsinger and Weir, 2009).

Phylogenetic trees were constructed using maximum-likelihood (MEGAX 10.18, Figure 7) and Bayesian (Mr. Bayes) methods. The maximum-likelihood trees are included here, which had similar topologies to the Bayesian trees. I selected the best model fit for each tree following model-testing with maximum-likelihood methods.

Genotyping-by-sequencing nuclear DNA laboratory methods

All mentioned samples were extracted for DNA using Qiagen DNA kits. The laboratory Data2Bio received extracted DNA from USDA Forest Service National Genomics Center for Wildlife and Fish Conservation laboratory and further concentrated the samples with low DNA. 52% of the samples had low DNA concentrations below 1.5 ng/uL, possibly due to limited available tissue from contamination removal. A de novo assembly was used as the reference genome for this project. Sample were sequenced using an Illumina HiSeq X instrument, a total of 2 x 311,900,753 reads was generated with an average of 6,115,701 reads per sample. Each individual sequence read was trimmed of low-quality regions and aligned to the reference genome using GNAP (Wu and Nacu, 2010). Only reads with a single unique alignment were used for analyses. A SNP was called as homozygous in a diploid sample if at least 5 reads supported the major common allele at that site and at least 90% of all aligned reads shared the same nucleotide at that site. A SNP was called as heterozygous in a diploid sample if at least 2 reads supported each of at least 2 different alleles and each of the 2 allele types separately made up more than 20% of the reads aligning to that site, and when the sum of the reads supporting those 2 alleles equaled 5 and made up at least 90% of all reads covering the site. The sites were

further filtered to obtain a set of 256,434 SNPs. 10 samples were removed during this filtering process due to high missing rate. These SNPs were further filtered by defining an acceptable minimum call rate (MCR) of > 50% per SNP. This dataset, MCR50, contained 194,575 SNPs from 41 samples. This dataset was further filtered to remove SNPs from outgroup species to create the dataset MCR50IN which had 101,253 SNPs from 35 *I. s. charlestonensis* samples.

Genotyping-by-sequencing Nuclear DNA analysis

To assess population structure in the MCR50IN population genetic dataset I used STRUCTURE (Pritchard et al., 2000), a Bayesian clustering approach that assesses population clusters that maximize Hardy-Weinberg equilibria. I ran 10 replicates of K = 1 – 10, each consisting of 100,000 generations of burn-in followed by 100,000 Markov-Chain Monte Carlo replicates. To determine the optimal value of ΔK I considered the Evanno method (Evanno et al., 2005) and ln Pr(X|K) from Pritchard et al. 2000, calculated with CLUMPAK v1.1 (Kopelman et al., 2015). To construct a phylogenetic tree from the MCR50 SNP dataset, pairwise distances were estimated between MCR50 individuals using an unbiased model of substitution frequencies. Neighbor-Joining algorithm was used to construct a phylogenetic tree using distance estimates (Saitou and Nei, 1987) and performed in the NJS module of the APE (Paradis et al., 2004) R package. The NJS algorithm is tolerant of missing data, making it useful for GBS data.

Results

Bayesian and maximum-likelihood phylogenetic analysis of mitochondrial DNA

The phylogenetic relationship of *Icaricia shasta charlestonensis I. s. Minnehaha, I. acmon, I. icarioides, and Euphilotes ancilla purpura* was created with a maximum likelihood model and 1000 bootstrap replicates. The best fit model was the Tamura-Nei model with a

discrete Gamma distribution and I nucleotide rate. The *I.s charlestonensis* haplotypes are monophyletic with slight divergences from the California *I. shasta* and Wyoming *I. s. minnehaha* subspecies (Figure 7; bootstrap support 64/65). In comparison, *Euphilotes ancilla* subspecies have a deeper divergence and higher bootstrap support (Figure 7; bootstrap support 100/99). 73 butterflies from four subpopulations of *Euphilotes ancilla purpura* in the Spring Mountains (Thompson et al., 2020) were used to compare these results. The *E. a. purpura* subset contained 8 mtDNA haplotypes that differed by a maximum of 8 mutational steps and the nucleotide diversity of the sample was only 0.0051 (Figure 8).

Haplotype network of mitochondrial DNA

A minimum spanning network of the 26 *I. s. charlestonensis* specimen collected from three locations the Spring Mountains revealed the connections within the subpopulations in the Spring Mountains. The haplotypes include eight unique mtDNA COI haplotypes and one divergent *I. s. minnehaha* haplotype. The samples were collected from South Loop Trail (n=10), Bonanza trail (n=9) and Lee Canyon ski area (n=7) with two haplotypes (B and D) making up 65% of the samples (B (n=6) and D (n=11)). Both haplotypes were found in all three locations, the D haplotype being the most frequent haplotype at South Loop and Bonanza Trail (Table 3). Haplotypes A, G, H, and I had one sample each within the Spring Mountains. From this small dataset, I observed two haplotypes occurring in all three subpopulations, indicating no genetic structuring.

Haplotype diversity and nucleotide diversity of mitochondrial DNA

The 8 haplotypes were relatively similar, differing by 1 to 5 bp changes (Figure 8). The maximum number of nucleotide differences between *I.s. charlestonensis* G and I was 5 (Figure

8). Comparatively, the minimum difference between *I. s. charlestonensis* haplotypes to *I. s. minnehaha* was 6 nucleotides from haplotypes H to F and D to F. While the maximum difference was 10 nucleotides from haplotype G to F (Figure 8). The gene (haplotype) diversity was 0.812 (± 0.057, S.D.; from DnaSP) with 9 segregating sites (Table 6). The overall nucleotide diversity was 0.00127 (± 0.00054, S.E.) with 9 segregating sites (Table 6). Given the lack of genetic structure shown in the mtDNA, the diversity in each of the three subpopulations is similar. Interestingly Lee Canyon ski area is the subpopulation with the least butterflies sampled and the highest nucleotide diversity ($\boldsymbol{\pi}$ = 0.001797). While South Loop Trail a main subpopulation, had the lowest nucleotide diversity ($\boldsymbol{\pi}$ = 0.001107; Table 6).

I used Analysis of Molecular Variance AMOVA to estimate subpopulation differentiation in COI haplotype frequencies with 3 subpopulations. AMOVA revealed no population structuring of the 8 mtDNA haplotypes and their frequencies in the 3 populations (Table 5). The AMOVA parameter measured the proportion of genetic diversity attributable to differences among subpopulations (Holsinger and Weir, 2009) at ΦST = 0.01, not significantly different from zero (Table 7). AMOVA showed a sum of squares among populations at 1.413 and within subpopulations at 14.267, genetic variance ranging from 0.007 – 0.658, and P, the significance level was 0.349 (Table 7). The percentage of genetic variance among subpopulations was only 1%, the rest from within subpopulations (99%) (Table 7).

The most common haplotypes B and D were present at Bonanza and South Loop Trail locations drove this parameter, along with all other haplotypes being at low frequency. Tajima's D was -1.397 in all the subpopulations, significantly less than zero. Other statistical measures of neutral equilibrium deviation like Fu's F (F = -2.563) Strobeck's S (S = 0.997) coincide with the interpretation of a moderately expanding population.

Population genetic analysis using nuclear sequence data

A total of 311,900,753 reads were obtained for an average of 6,115,701 reads per sample (range 395,922 – 83,570,085). A smaller data set MCR50 had 194,575 SNPs. This dataset was further filtered to remove SNPs from outgroup species to create the dataset MCR50IN which had 101,253 SNPs. I used GenAlEx to analyze the first 8,190 SNP loci. This dataset shows some population structure in the *I. s. charlestonensis* subspecies. The mean FST value, representing genetic variance across the populations is 0.094. Gene flow across the 4 populations is Nm = 6.33. The average number of alleles per SNP locus from each cluster ranged from 1.714 – 1.808 (Table 9). The number of effective alleles from each cluster ranged from 1.406 – 1.441. The Shannon's information index from each cluster ranged from 0.370 – 0.402. The observed average heterozygosity (Ho) from each population ranged from 0.224 – 0.260, where the South Loop cluster had the lowest Ho (Table 9). Pairwise Fst between the four clusters ranged from 0.047 – 0.080 (Table 10). Observed heterozygosity ranged from 0.224 – 0.249 (Table 9), the lowest Ho being from the South Loop population.

In the STRUCTURE analysis ΔK supported K = 3 and K = 4 (Figure 9). Both K = 3 and K = 4 identifies the South Loop population as a distinct cluster with little mixing with other populations. LnPr(K|X) strongly supported K =3 and the Evanno method supported K = 4.

Visually I can see the Lee Canyon butterflies have the most variation and mixing of SNPs at K = 3 and K = 4. The South Sister and Bonanza butterflies show low levels of variation at K = 3 and increased variation at K = 4 with the introduction of the green cluster.

Principal component analysis (PCA) was conducted on the 8190 SNP dataset to compare the consistency of results obtained using GenAlEx (Peakall and Smouse, 2006) to calculate

genetic distances and convert to a covariance matrix with data standardization. The PCA shows the Lee Canyon, South Sister, and Bonanza populations clustering in both PCA 1 vs 2 and PCA 2 vs 3. This shows that those three Lee Canyon populations are more similar to each other than the South Loop population, which is has less overlap with the other populations. Principle component analysis supports evidence that South Loop population is genetically differentiated from the three other populations. Notice that the South Loop population has much less overlap with the 3 other populations across the Spring Mountains. The grey triangles representing the South Loop population, are not clustering together with the other three populations (Figure 10). PC 1 vs 2 South Look has little overlap with South Sister and Bonanza populations. While Bonanza and Lee Canyon populations cluster heavily. The first three components of PCA explained 21.68% of the total variance (component 1: 11.67%, component 2 5.47%, component 3: 4.54%). Overall, PCA showed similar broadscale patterns to the other analyses: the Lee Canyon, South Sister, and Bonanza populations clustering in both PCA 1 vs 2 and PCA 2 vs 3, suggesting that gene flow may be highest among sites that are geographically most proximate.

The more robust MCR50IN dataset shows overall Ho = 0.243, heterozygosity within populations Hs = 0.277, total heterozygosity Ht = 0.288, corrected total heterozygosity H’t = 0.291, and the inbreeding coefficient Gis = 0.124 (Table 11; Nei, 1987). The number of alleles in each cluster ranged from 1.755 – 1.804 (Table 12). The number of effective alleles and Ho was similar to the smaller dataset, ranging from 1.406 – 1.434 and 0.223 – 0.258 respectively. The inbreeding coefficient ranged from 0.068 – 0.156, the highest being from the South Loop cluster. Pairwise Fst in the MCR50IN dataset ranged from 0.005 to 0.127, the highest being from the South Loop cluster.

The phylogenetic tree from the MCR50 SNP dataset (Figure 11) shows *I. s. charlestonensis* individual collected from subpopulations across the Spring Mountains. The tree groups the South Loop cluster monophyletically. Lee Canyon is grouped paraphyletically with one individual grouping closer to Bonanza butterflies. Bonanza, South Sister, and Lee Canyon are mixed with each other, with Bonanza and South Sister showing the most mixing. The outgroup species are grouped together along with one Bonanza individual. The outgroup species include *Icaricia shasta minehaha*, *Plebejus acmon*, *Icaricia icarioides austinorum*, and *Icaricia icarioides austinorum.*

Discussion

My characterization of a dynamic butterfly metapopulation demonstrates the evolutionary patterns of divergence at a fine spatial scale in a restricted range. *I. s. charlestonensis* has a range limited to the geographic distribution of resources for which it has evolved. The Spring Mountains in Southern Nevada where *I. s. charlestonensis* reside can be considered a sky island, because they are isolated from mountain ranges of similar size by 80 miles of low elevation desert (Austin, 1981). *I. s. charlestonensis* habitat is only found at elevations above 2,500 meters (Weiss et al., 1997). Specialized resources requirements including non-consumable larval host plants for reproduction and consumable nectar plants further restrict its range to the distribution of those resources. These small-scale geographic restrictions have led to population and genetic consequences in other species (Brown, 1984; Lester et al., 2007; Nolte et al., 2019). I conducted a genetic assessment of populations of *I. s. charlestonensis* across the Spring Mountains to determine if small-scale geographic restrictions in specialized habitat cause small-scale phylogeographic structure and genetic isolation within it's already restricted range. My results gave evidence for resource restricted populations that led to genetic divergence and population

differentiation in the South Loop trail population as well as interconnected subpopulations in Lee Canyon. While these results have conservation implications, they also give us a glimpse into the mechanisms of genetic divergence, especially for animals with low vagility and host specificity.

Mitochondrial DNA analyses

I hypothesized that there would be little or no genetic divergence across the Spring mountains, and that the genetic diversity of the subpopulations would not be significantly different. The evidence from the mitochondrial DNA supported this hypothesis and suggested *I. s. charlestonensis* is a metapopulation with enough gene flow and recolonization of subpopulations to offset genetic divergence (Thompson, 2017). The 26 *I. s. charlestonensis* collected from the Spring Mountains yielded 8 unique mtDNA COI haplotypes. Two of those haplotypes (B and D) were present in all 3 subpopulations and made up 65% of the samples, indicating little genetic structuring in the mtDNA dataset. In addition, the nucleotide diversity in each of the three populations was similar ($\boldsymbol{\pi}$ = 0.00104 – 0.0018; Table 6), which would be expected in populations that lack genetic structure. The haplotypes only differed by 1 to 5 base pairs, revealing their similarity to each other (Figure 8). Similarly, the *I. s. minnehaha* to *I. s. charlestonensis* haplotype had a low minimum difference of six mutational steps (Figure 8). Further evidence from the mtDNA for little divergence came from the AMOVA results, showing the among subpopulation genetic variance at only 1%, while 99% was from within subpopulation genetic variance (Table 7). The AMOVA results from 26 *I. s. charlestonensis* COI haplotypes among South Loop Trail, Bonanza trail, and Lee Canyon SA was based on 9,999 permutation. AMOVA showed a sum of squares among populations at 1.413 and within subpopulations at 14.267, genetic variance ranging from 0.007 – 0.658. The percentage of genetic variance among subpopulations was only 1%, the rest from within subpopulations (99%). Further, the parameter

ΦST was 0.0101 (Table 7), suggesting that the genetic diversity attributable to differences among subpopulations was not significantly different from zero.

The higher nucleotide diversity in Lee Canyon ski area ($\boldsymbol{\pi}$ = 0.001797) gives some evidence of higher level of mixing of individuals in the Lee Canyon ski area from the Bonanza and South Sister subpopulation. The lowest nucleotide diversity in South Loop Trail ($\boldsymbol{\pi}$ = 0.001107; Table 6) is likely explained by the geographic separation of the locale, leading to less gene flow, and mixing of individuals. Haplotype diversity was 0.812 (± 0.057, S.D; from DnaSP) with 9 segregating sites. Such a high level of haplotype diversity suggests there is a current large effective population size and low genetic drift.

Tajima's D was -1.397 in all the subpopulations, significantly less than zero. The negative D (difference statistic) presents the excess of rare segregating sites that would be expected for the level of nucleotide diversity in the subpopulations. This shows a deviation from a neutrally evolving population and indicative of an expanding population (Tajima 1989). Other statistical measures of neutral equilibrium deviation like Fu's F (F = -2.563) Strobeck's S (S = 0.997) coincide with the interpretation of a moderately expanding population. Population growth and excess segregating sites occur on an evolutionary time scale with the timing of expansion based on mutation rates and neutral processes (Tajima 1989; Fu 1996; Ramirez-Soriano et al., 2008). It is important to note that this result is based on a small sample size and should therefore not be asserted since small sample size and use of a single marker likely produces unreliable results. However, the results of the first genetic data showed no evidence of a loss of genetic diversity in *I. s. charlestonensis*.

The mtDNA implied that *I.s. charlestonensis* is one large metapopulation with gene flow among populations to counteract genetic divergence that could lead to local extinction.

Mitochondrial DNA alone has limitations, one being that mtDNA represents only a single locus. It gives us a small glimpse into the evolution of a species, and only reflects the matrilineal history which can differ from the overall population history. One explanation of subpopulation differentiation with shared mtDNA haplotypes would be that South Loop has low or no gene flow yet shares similar genetic markers due to incomplete lineage sorting (Marko and Hart 2011). Despite the evidence for effectively one population, genetic population structure based on the SNP dataset were not entirely consistent with the mtDNA dataset, supporting evidence that single locus datasets should be interpreted with caution.

Population genetic analysis using nuclear sequence data

The results from the SNP analysis indicate that there is evidence of genetic divergence and population differentiation. The distances separating the South Loop Trail subpopulation from the Lee Canyon, Bonanza Trail, and South Sister Trail locations (a minimum direct line separation of approximately 2.5 miles) acts as a geographic barrier to gene flow. I found that *I. s. charlestonensis* consists of an isolated subpopulation with low levels of gene flow that allowed for genetic divergence, in addition to a slightly structured metapopulation with enough gene flow from subpopulation habitat areas to offset neutral genetic divergence. This conclusion partially supports both of my hypotheses. In the Lee Canyon, Bonanza Trail, and South Sister Trail locations I observed more mixing subpopulations, supporting my prediction that there would be little or no genetic divergence across the Spring Mountains. Whereas in the South Loop location there was more divergence likely due to geographic and resource restrictions supporting my hypothesis that the fragmented, patchy distribution of larval host plants would cause modest to moderate genetic divergence among subpopulations based on observations of limited and highly localized movements of adults. Initially the mtDNA led me to believe that butterflies were using

patches of habitat to migrate across the drainage slope (Andrew et al. 2013; Thompson et al. 2017; Thompson 2018). Other possible modes of migration include dispersal by wind (Thompson et al. 2014; Gradish and Otis, 2015) or positive density-dependent emigration, the emigration of butterflies when carrying capacity is exceeded (Nowicki and Vrabec, 2011). However, the SNP data suggests migration is occurring between the locations excluding South Loop. This gives evidence that lack of resources (host plants and larval plants) between Lee Canyon and Kyle Canyon is restricting *I. s. charlestonensis* dispersal.

Alternatively, if the carrying capacity of butterfly locales has not been reached, butterflies from South Loop would not exhibit positive density-dependent dispersal across the Spring Mountains. However, *Icaricia* butterfly behavior has been observed leaving larval host plant areas at the edge of habitat areas in addition to persisting in areas where large burns have occurred, implying the dispersal of adults and larvae over large areas that include unburned habitat. This suggests that *Icaricia* leave habitat areas at population densities below the carrying capacity of the habitat. At the South Loop location in 2020 and 2021, butterflies have been observed 0.5 miles into the burned habitat following the fire in 2013 (Thompson, 2022). Observations of another *Icaricia* species indicate that blue butterflies bias their flight to stay within their habitat but leave habitat areas at an observable rate (Schultz, 1998), indicating that they change their behavior based on the habitat that is present. However, a review of lycaenid butterflies showed that out of 5 species only 1 traveled over 2 km (Hanski and Kuussaari, 1995). The > 4 km distance from South Loop to Bonanza Trail and Lee Ski habitat would be a great distance for *I. s. charlestonensis* to travel, and due to the quality and large size of South Loop habitat and behavior patterns, butterflies would be unlikely to travel that distance. Unless there were patches of habitat within short distances (<10 m) (Schultz, 1998), producing stepping-stone

connections across the Kyle Canyon drainage, the South Loop population will remain isolated from other habitat areas. It appears likely that, given the genetic patterns observed, stepping stone patches of habitat have not existed for *I. s. charlestonensis* between the South Loop and Lee Canyon populations within recent evolutionary time frames. Future research could focus on searching for larval host plant patches in the Kyle Canyon drainage area to confirm that lack of resources is the cause of the current genetic isolation of populations. The use of high-powered optics, drones or helicopters would be highly recommended to locate plants on the steep terrain.

In the past, the geography of the Spring Mountains allowed for the dispersal of *I. s. charlestonensis* from South Loop area to Lee Canyon area, likely due to the absence of geographic barriers; high exchange of alleles within the population would have maintained genetic similarity across locales and maintained the subspecies. At some point in the evolutionary history of *I. s. charlestonensis*, the lack of essential resources and the geographic distance between populations posed a barrier to gene flow and led to a lack of migration from South Loop to Lee Canyon habitat. Apparently enough time has passed to allow for neutral genetic divergence of these populations. If there continues to be a lack of gene flow between South Loop and the other *I. s. charlestonensis* populations, over time, genetic drift could lead to sub-species level divergence or even speciation. It is difficult to predict the number of butterflies that need to migrate from one population to another to maintain genetic connectivity. Slatkin (1987) is often cited as stating one migrant per generation will prevent drift, however, that number is a rough estimate and depends on other factors such as the source population and effective population size (Nathan et al., 2017).

In contrast, the Lee Canyon populations have had intermediate level of subdivision with some level of migration occurring between populations. This type of population subdivision

would generally yield the highest adaptive potential with possibilities of local adaptation to local environments, where there is occasional gene flow and a large enough effective population to prevent inbreeding and the loss of genetic variation. Therefore, conservation efforts must focus on maintaining habitat that can sustain population sizes large enough to maintain high levels genetic variation.

Given the observations that the Lee Canyon ski area had low population numbers from 2008 – 2018 (Thompson, 2014), I would expect that in the absence of gene flow the ski area would have lower genetic diversity parameters than Bonanza, a more stable population. The Lee Canyon populations (Lee Canyon ski area, Bonanza Trail, South Sisters Trail) all have comparable genetic diversity parameters, except for the inbreeding coefficient, Gis, at the Lee Canyon ski area. The latter's Gis, is approximately half the Gis of the other subpopulations (Table 12). One might expect that because Bonanza harbors a large and stable population that it would have the lowest Gis in comparison to Lee and South Sister who had low butterfly numbers from 2004 to 2018 (Thompson, 2018). South Sister is a much smaller habitat area than Lee Canyon, yet diversity parameters are comparable to the Bonanza Trail population. Small populations generally lead to an accelerated loss of genetic variation due to genetic drift, unless counteracted by the introduction of new alleles from migration into the population (Allendorf, 2012). Because the genetic diversity parameters at Lee Canyon ski area are comparable to Bonanza, despite the low population numbers, I can conclude genetic diversity at Lee Canyon ski area must be the result of extensive butterfly migration into low elevation in 2019 and 2020, perhaps due to positive density-dependent emigration. This observation is supported by the low inbreeding parameter, which is lower than would be expected in a small population with little migration (Table 12). From this I can infer the migration directionality of *I. s. charlestonensis* is

from the higher elevation population at Bonanza Trail to the lower elevation population at Lee Canyon ski area.

The endangered *I. s. charlestonensis* displayed genetic differentiation with the South Loop population divergent from the other three populations in the Spring Mountains. The geographic distances separating the South Loop Trail subpopulation from the Lee Canyon, Bonanza, and Sisters subpopulation is approximately 4 km. *I. s. charlestonensis* habitat spans less than 11 km, yet clearly show genetic structure. While the mtDNA analysis suggested a lack of subpopulation structure, the larger SNP dataset clearly showed a genetically differentiated South Loop population. The SNP data showed Fst = 0.094, an intermediate genetic differentiation value below 0.15, what Wright (1978) calls a 'great' genetic differentiation value. Fst values above 0.15 are genetically distinct enough to be considered as evolutionarily significant units (Frankham 2002). Lowe and Allendorf (2010) suggest Fst values >0.2 lead to inbred populations and Fst values >0.35 prevent the sharing of advantageous alleles between populations (adaptive connectivity). Pairwise Fst between the four clusters ranged from 0.047 – 0.080 (Table 9). The South Loop has the highest pairwise Fst to other clusters (Table 10), supporting STRUCTURE evidence that South Loop is the most distinct of these grouping in K=3 and K=4. All the pairwise Fst value display an intermediate genetic differentiation value below Wright's 0.15 'great' genetic differentiation value (1978). The overall Fst was 0.094, a value greater than the pairwise differences. Observed heterozygosity ranged from 0.224 – 0.249 (Table 9), the lowest Ho being from the South Loop population.

Genetic drift within each subpopulation acts to increase divergence, Fst, among subpopulations. However, gene flow (migration) between subpopulations acts to decrease Fst (Wright, 1969). Nm was equal to 6.33, greater than Nm = 1.0, suggesting there is gene flow

negating genetic drift and maintaining connected populations. Out of the four locations, I can observe gene flow occurring when there is a mixing of genes. This is most prevalent in the Lee Canyon ski area subpopulation, followed by South Sister and Bonanza Trail subpopulations (Figure 9). The three Lee Canyon populations (South Sister, Lee Canyon ski area, and Bonanza) likely contribute to most of the gene flow between populations. The South loop population is geographically separated from the other three populations, a minimum direct line separation of approximately 2.5 miles.

The smaller 8190 SNPs dataset showed gene flow across the 4 populations as Nm = 6.33. Nm values >1 theoretically indicate a lower likelihood of genetic drift, thus limiting the genetic differentiation among populations. Because I can observe three populations having moderate gene flow and mixing, and the south loop population having very little mixing (Figure 9), I know that the South Loop subpopulation is pulling the average level of gene flow down, while the other three populations are maintaining that higher geneflow level. The South Loop cluster had the lowest observed average heterozygosity and number of effective alleles in the smaller SNP dataset (Table 9). Unexpectedly, South Sister had the lowest average number of alleles per SNP locus and Shannon's Index (Table 9). This could be due to the low number of SNPs analyzed. Genetic differentiation in the South Loop population is also supported in the MCR50IN dataset, which shows South Loop with the lowest level of effective alleles and heterozygosity while having the highest inbreeding coefficient (Table 12) and pairwise Fst (Table 13).

The phylogenetic tree (Figure 11) shows well supported monophyletic clade including South Loop individuals corresponding to the South Loop cluster. The Lee Canyon clade forms a monophyletic clade that includes all individuals corresponding to the Lee Canyon cluster except for one individual that is grouped closer to Bonanza butterflies. Besides these clades, there are

clusters of subpopulations although they are paraphyletic (Figure 11). Bonanza shares a clade with each of the three subpopulations. The outgroup species are grouped together monophyletically along with one Bonanza individual.

Threats, stressors, and conservation implications

The major threats and stressors to *I. s. charlestonensis* are the loss and degradation of habitat due to forest succession and changes in natural fire regime, grass or shrub overgrowth and non-native plants, development or fuels reduction projects, disturbances by recreation activity and feral horse activity (Thompson, 2018). Climate models predict increased drought and extreme precipitation events that can exacerbate these threats. The most frequent stressor identified by USFWS habitat assessments were increased tree canopy encroachment through forest succession and change or fire regime, physical disturbance of host plants by feral horses, and overgrowth of host plants by native grasses or non-native plants. There is likely an interaction between these stressors, since feral horse grazing in the Lee Canyon ski area has diminished overgrowth of host plants by native grasses and non-native plants.

Butterfly abundance and habitat use has revealed high variability from year to year (Thompson, 2018; Andrews et al., 2013). Maintenance or enhancement of habitat in the Spring Mountains with increased nectar plant abundance will serve to facilitate butterfly movement. Recommended nectar plants for out planting include *Erigeron clokeyi*, *Hymenoxys cooperi*, *Eriogonum umbellatum* var *versicolor and Gutierrezia sarothrae*. Enhancement in small patches of habitat can serve as stepping stones for butterflies into unoccupied habitat during years of high abundance (Thompson, 2022; Crawford, 2011).

The disruption of gene flow due to restrictions can cause isolation and increase the risk of extinction (Allendorf, 2012; Nolte et al., 2019). However, the recent discovery of high elevation habitat, particularly the relatively large and recovered South Loop habitat, has shown *I. s. charlestonensis* to be more abundant than previously believed. As South Loop habitat continues to recover from fire damage, butterfly habitat will continue to increase. Notwithstanding local extinctions due to habitat conditions worsening. In the case of increased threats to the subspecies, more out planting should be considered, along with movement of butterflies or eggs into unoccupied, suitable habitat areas. Current estimates of population structure suggest butterflies can be drawn from the high elevation populations in the Bonanza and South Sister populations without concern for differences in genetic diversity. Considering South Loop's genetic differentiation, isolation is not so extensive as to warrant it a separate evolutionary unit (Frankham 2002). However, the South Loop subpopulation has moderate levels of genetic differentiation and therefore should only be used for genetic rescue if no other individuals from Lee Canyon subpopulations are available.

Concluding remarks

Revealing the genetic structure of *I. s. charlestonensis* and *P. giulianii* illustrates how specialization and isolation can affect threatened insect populations and highlights how fine-scale studies can reveal patterns of evolution. By using genetic data, I was able to show that both *P. giulianii* and *I. s. charlestonensis* exhibit fine-scale fragmentation with exceptionally limited distributions of restricted resources. The geographic scale of the studied regions are extremely limited, and yet both analyses uncovered evidence of spatial genetic structure. Genetic data from *P. giulianii* revealed that there is genetic divergence and population differentiation due to limitations in the geographic distribution of essential resources associated with sand substrates.

The SNP data also revealed phylogenetic divergence between *P. giulianii* populations from Big Dune and Lava Dune at a fine geographic scale. The nuclear data revealed a divergence between the *I. s. charlestonensis* population from South Loop and the rest of the Spring Mountains due to resource restriction. Additionally, little is known about the migration patterns of *I. s. charlestonensis*, my study showed it is likely high elevation *I. s. charlestonensis* migrate to lower elevations and that there is very limited dispersal either into or out of the South Loop population. Despite the Lee Canyon ski area being very disturbed by the ski activity, there is maintenance of high levels of genetic diversity. Investigating the use of small patch habitat would be useful to understand the dispersal ability and connectivity among populations of *I. s. charlestonensis*. Future studies should detail which environmental factors facilitate genetic differentiation in both species.

Appendix

Table 1. Coleoptera sample list. Species, collection location, number of beetles used for mtDNA analysis, identification label from NCBI. *Pseudocotalpa giulianii* specimen used to find haplotypes for figure 4.

Species	Collection location	# of beetles	ID
Outgroup			
Chrysina aurigans	NCBI	1	Chrysina aurigans isolate 336 (COI) gene, partial cds; mitochondrial.fasta
Chrysina aurilisternum	NCBI	1	Chrysina aurilisternum isolate C09 (COI) gene, partial cds; mitochondrial.fasta
Chrysina bruyeai	NCBI	1	Chrysina bruyeai isolate 334 (COI) gene, partial cds; mitochondrial.fasta
Chrysina limbata	NCBI	1	Chrysina limbata isolate 333 (COI) gene, partial cds; mitochondrial.fasta
Chrysina luteomarginata	NCBI	1	Chrysina luteomarginata isolate 335 (COI) gene, partial cds; mitochondrial.fasta
Chrysina peruviana	NCBI	1	Chrysina peruviana isolate Cp05 (COI) gene, partial cds; mitochondrial.fasta
Cotalpa lanigera	NCBI	1	Cotalpa lanigera (COI) GU013589.1
Paracotalpa ursina	NCBI	1	Paracotalpa ursina (CO1) MG099691.1
Ingroup			
Pseudocotalpa andrewsi	Algodones Dune recreation area, CA	32	
Pseudocotalpa sonorica	Algodones Dune wilderness area, CA	22	
Pseudocotalpa giulianii	Amargosa Valley, Big Dune, NV	91	
Pseudocotalpa giulianii	Amargosa Valley, Lava Dune, NV	49	

Table 2. Coleoptera specimen used for SNP analysis. Species, genetic label, state beetle was collected, Latitude (degrees, minutes), Longitude (degrees, minutes).

Species	Genetic label	State	Location	Latitude	Longitude
Pseudocotalpa giulianii	LD6_2020	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD22_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD36_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD26_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD15_2020	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD8_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD14_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD4_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD2_2020	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD13_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD16_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD18_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD21_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'

Table 2.
Continued.

Species	Genetic label	State	Location	Latitude	Longitude
Pseudocotalpa giulianii	LD25_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD17_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD24_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD5_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD6_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD20_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD2_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD7_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD13_2020	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD1_2020	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD9_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD19_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD1_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD12_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'

Table 2.
Continued.

Species	Genetic label	State	Location	Latitude	Longitude
Pseudocotalpa giulianii	LD4_2020	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD3_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD23_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD33_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD11_2020	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD31_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD15_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD9_2020	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD10_2020	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD34_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD3_2020	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD7_2020	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD14_2020	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD5_2020	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'

Table 2.
Continued.

Species	Genetic label	State	Location	Latitude	Longitude
Pseudocotalpa giulianii	LD35_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD8_2020	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD28_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD12_2020	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD29_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD27_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD30_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	LD32_2021	NV	Amargosa Valley, Lava Dune	N 36° 41.2'	W 116° 29.9'
Pseudocotalpa giulianii	BD39_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD20_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD30_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD16_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD26_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD38_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'

Table 2.
Continued.

Species	Genetic label	State	Location	Latitude	Longitude
Pseudocotalpa giulianii	BD14_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD30_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD23_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD7_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD35_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD34_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD41_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD39_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD40_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD17_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD19_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD11_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD4_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD21_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'

Table 2.
Continued.

Species	Genetic label	State	Location	Latitude	Longitude
Pseudocotalpa giulianii	BD16_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD37_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD20_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD22_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD42_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD27_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD21_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD29_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD14_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD4_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD19_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD17_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BDU1_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD12_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'

Table 2.
Continued.

Species	Genetic label	State	Location	Latitude	Longitude
Pseudocotalpa giulianii	BD15_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD15_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD7_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD24_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD22_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD35_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD2_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD13_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD18_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD36_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD40_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD3_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD37_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD9_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'

Table 2.
Continued.

Species	Genetic label	State	Location	Latitude	Longitude
Pseudocotalpa giulianii	BD43_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD38_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD10_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD12_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD13_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD24_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD3_2020	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD42_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Pseudocotalpa giulianii	BD45_2021	NV	Amargosa Valley, Big Dune	N 36° 39.28'	W 116° 34.9'
Cotalpa flavida	OG1_2022	NV	Moapa	N 36° 44'	W 114° 39'
Pseudocotalpa sonorica	SMVD2	CA	Algodones Dune recreation area	N 32° 59.31'	W 115° 08.10'
Pseudocotalpa andrewsi	A7_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa sonorica	S14_2022	CA	Algodones Dune recreation area	N 32° 59.31'	W 115° 08.10'
Pseudocotalpa andrewsi	A9_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'

Table 2.
Continued.

Species	Genetic label	State	Location	Latitude	Longitude
Pseudocotalpa andrewsi	A6_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa andrewsi	A2_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa andrewsi	AR3_2021	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa andrewsi	A16_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa andrewsi	AR5_2021	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa andrewsi	A3_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa andrewsi	A17_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa andrewsi	AR4_2021	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa andrewsi	A14_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa andrewsi	AR1_2021	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa andrewsi	A13_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa sonorica	S12_2022	CA	Amargosa Valley, Lava Dune	N 32° 59.31'	W 115° 08.10'
Pseudocotalpa andrewsi	A15_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W114° 59.056'
Pseudocotalpa andrewsi	A5_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'

Table 2.
Continued.

Species	Genetic label	State	Location	Latitude	Longitude
Pseudocotalpa andrewsi	A12_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa andrewsi	A1_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa andrewsi	A11_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa andrewsi	A10_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa sonorica	S10_2022	CA	Amargosa Valley, Lava Dune	N 32° 59.313'	W 114° 59.056'
Pseudocotalpa andrewsi	A4_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa andrewsi	A8_2022	CA	Amargosa Valley, Lava Dune	N 32° 55.177'	W 114° 59.056'
Pseudocotalpa sonorica	S7_2022	CA	Amargosa Valley, Lava Dune	N 32° 59.31'	W 115° 08.10'
Pseudocotalpa sonorica	SAU3_2021	CA	Amargosa Valley, Lava Dune	N 32° 59.31'	W 115° 08.10'
Pseudocotalpa sonorica	S9_2022	CA	Amargosa Valley, Lava Dune	N 32° 59.31'	W 115° 08.10'
Pseudocotalpa sonorica	SMB9_2021	CA	Amargosa Valley, Lava Dune	N 32° 59.31'	W 115° 08.10'
Pseudocotalpa sonorica	S13_2022	CA	Amargosa Valley, Lava Dune	N 32° 59.31'	W 115° 08.10'
Pseudocotalpa sonorica	S3_2022	CA	Amargosa Valley, Lava Dune	N 32° 59.31'	W 115° 08.10'

Table 2.
Continued.

Species	Genetic label	State	Location	Latitude	Longitude
Pseudocotalpa sonorica	S15_2022	CA	Amargosa Valley, Lava Dune	N 32° 59.31'	W 115° 08.10'
Pseudocotalpa sonorica	S8_2022	CA	Amargosa Valley, Lava Dune	N 32° 59.31'	W 115° 08.10'
Pseudocotalpa sonorica	S4_2022	CA	Amargosa Valley, Lava Dune	N 32° 59.31'	W 115° 08.10'
Pseudocotalpa sonorica	S6_2022	CA	Amargosa Valley, Lava Dune	N 32° 59.31'	W 115° 08.10'
Pseudocotalpa sonorica	S11_2022	CA	Amargosa Valley, Lava Dune	N 32° 59.31'	W 115° 08.10'
Pseudocotalpa sonorica	S1_2022	CA	Amargosa Valley, Lava Dune	N 32° 59.31'	W 115° 08.10'
Pseudocotalpa sonorica	S5_2022	CA	Amargosa Valley, Lava Dune	N 32° 59.31'	W 115° 08.10'
Pseudocotalpa sonorica	S2_2022	CA	Amargosa Valley, Lava Dune	N 32° 59.31'	W 115° 08.10'

Table 3. Estimates of genetic diversity for 140 COI mitochondrial DNA sequences of *Pseudocotalpa giulianii* among two sample locations and the total of both populations. Parameter estimates for n = number of sequences, Hn = number of haplotypes, Hd = Haplotype diversity, π = nucleotide diversity, S = Number of segregating sites.

Location	N	Hn	Hd	π	S
All populations	140	5	0.57708	0.00185	5
Big Dune	91	4	0.33529	0.00064	4
Lava Dune	49	2	0.25000	0.0086	2

Table 4. Genetic differentiation (Fst) and gene flow (Nm) among *Pseudocotalpa giulianii* populations.

Location	Fst	Nm
Big Dune / Lava Dune	0.76650	0.08

Table 5. Locations of eight *Icaricia shasta charlestonensis* mtDNA COI haplotypes (A – E, G – I) and one *Icaricia shasta minnehaha* sampled from Wyoming.

Subspecies	***I. s. charlestonensis***								***I. s. minnehaha***	
Haplotypes	**A**	**B**	**C**	**D**	**E**	**G**	**H**	**I**		**F**
South Loop Trail		2	1	5	2					
Bonanza Trail		2	1	4			1	1		
Lee Canyon Ski	1	2		2	1	1				
Total	**1**	**6**	**2**	**11**	**3**	**1**	**1**	**1**		**1**

Table 6. Estimates of genetic diversity for 26 mtDNA haplotype samples of *Icaricia shasta charlestonensis*. Estimates for m = number of sequences, n = total number of sites, S = Number of segregating sites, ps = S/n, Θ = ps/a1, π = nucleotide diversity, and H is the number of haplotypes.

Location	***m***	***S***	p_s	***Θ***	***π***	**H**
All populations	26	9	0.0074	0.0019	0.00127 $\pm$0.00054	8
South Loop Trail	10	4	0.00245	0.00087	0.00104	4
Bonanza Trail	9	5	0.0041	0.0014	0.001107	5
Lee Canyon SA	7	6	0.0049	0.0021	0.001797	5

Table 7. AMOVA of 26 *Icaricia shasta charlestonensis* mtDNA COI haplotype samples among 3 sample locations. d.f. = Degrees of freedom, SS = Sum of Squares, Estimated genetic variance, Percentage of genetic variance %, ΦST and P, the significance level based on 9,999 permutations.

Source of variation	**d.f.**	**SS**	**Estimated variance**	**Percentage of variance**	**ΦST** ***Phi-ST***	**P**
Among Subpopulations	2	1.413	0.007	1%	0.0101	0.349
Within Subpopulations	23	14.267	0.648	99%		
Total	25	15.680	0.656	100%		

Table 8. *Icaricia shasta charlestonensis* used in MCR50IN SNP nuclear DNA analysis.

Species	Sample name	Location
Icaricia shasta charlestonensis	B12	Lee Canyon SA
Icaricia shasta charlestonensis	B33	Lee Canyon SA
Icaricia shasta charlestonensis	B42	Lee Canyon SA
Icaricia shasta charlestonensis	B45	Lee Canyon SA
Icaricia shasta charlestonensis	B46	Lee Canyon SA
Icaricia shasta charlestonensis	T1	Lee Canyon SA
Icaricia shasta charlestonensis	T2	Lee Canyon SA
Icaricia shasta charlestonensis	B34	Lee Canyon SA
Icaricia shasta charlestonensis	B47	Lee Canyon SA
Icaricia shasta charlestonensis	BZ43	Bonanza
Icaricia shasta charlestonensis	BZ44	Bonanza
Icaricia shasta charlestonensis	D6	Bonanza
Icaricia shasta charlestonensis	BZ40	Bonanza
Icaricia shasta charlestonensis	BZ46	Bonanza
Icaricia shasta charlestonensis	BZ41	Bonanza
Icaricia shasta charlestonensis	BZ42	Bonanza
Icaricia shasta charlestonensis	D2	Bonanza
Icaricia shasta charlestonensis	M11	Bonanza
Icaricia shasta charlestonensis	R3	South Loop
Icaricia shasta charlestonensis	R4	South Loop
Icaricia shasta charlestonensis	R2	South Loop

Table 8. Continued.

Species	Sample name	Location
Icaricia shasta charlestonensis	SLWR 21	South Loop
Icaricia shasta charlestonensis	SLWR 47	South Loop
Icaricia shasta charlestonensis	R1	South Loop
Icaricia shasta charlestonensis	R5	South Loop
Icaricia shasta charlestonensis	SLWR 23	South Loop
Icaricia shasta charlestonensis	SLWR 24	South Loop
Icaricia shasta charlestonensis	SLWR 45	South Loop
Icaricia shasta charlestonensis	SLWR 20	South Loop
Icaricia shasta charlestonensis	S10	South Sister
Icaricia shasta charlestonensis	S24	South Sister
Icaricia shasta charlestonensis	S20	South Sister
Icaricia shasta charlestonensis	S21	South Sister
Icaricia shasta charlestonensis	S23	South Sister
Icaricia shasta charlestonensis	S22	South Sister

Table 9. Populations sampled and descriptive population genetic statistics using 8190 SNPs from nuclear DNA. N: number of individuals, Na: average number of alleles per SNP locus, Ne: number of effective alleles, I: Shannon's information index, Ho: observed average heterozygosity.

Population		**N**	**Na**	**Ne**	**I**	**Ho**
Lee Canyon ski area	Mean	7.615	1.783	1.428	0.390	0.260
	SE	0.015	0.005	0.004	0.003	0.002
Bonanza	Mean	7.522	1.808	1.441	0.402	0.249
	SE	0.016	0.004	0.004	0.003	0.002
South Loop	Mean	8.381	1.759	1.406	0.372	0.224
	SE	0.024	0.005	0.004	0.003	0.002
South Sister	Mean	4.978	1.714	1.409	0.370	0.242
	SE	0.012	0.005	0.004	0.003	0.003

Table 10. Pairwise FST for *Icaricia shasta charlestonensis* made using 8190 SNPs from nuclear DNA.

Pairwise Population Fst Values				
Lee Canyon	**Bonanza**	**South Sister**	**South Loop**	
0.000	--	--	--	**Lee Canyon**
0.047	0.000	--	--	**Bonanza**
0.074	**0.069**	0.000	--	**South Sister**
0.061	0.052	**0.080**	0.000	**South Loop**

Table 11. Summary of indices of genetic diversity for MCR50IN nuclear DNA SNP dataset. N: number of alleles, Ne: number of effective alleles, Ho: observed heterozygosity, Hs: heterozygosity within populations, Ht: total heterozygosity, H't: corrected total heterozygosity, Gis: inbreeding coefficient.

Statistic	Value	Standard Deviation	CI 2.5%	CI 97.5%
N	1.999	0.000	1.999	1.999
Ne	1.388	0.001	1.386	1.389
Ho	0.243	0.000	0.242	0.244
Hs	0.277	0.000	0.276	0.278
Ht	0.288	0.001	0.287	0.289
H't	0.291	0.001	0.290	0.292
Gis	0.124	0.001	0.121	0.126

Table 12. Populations sampled and descriptive population genetic statistics using SNPs from MCR50IN nuclear DNA dataset. N: number of alleles, Ne: number of effective alleles, Ho: observed heterozygosity, Hs: heterozygosity within populations, Gis: inbreeding coefficient.

Indices of genetic diversity per population					
Population	**N**	**Ne**	**Ho**	**Hs**	**Gis**
Lee Canyon	1.783	1.429	0.258	0.277	0.068
Bonanza	1.804	1.434	0.246	0.284	0.132
South Loop	1.755	1.406	0.223	0.265	0.156
South Sister	1.717	1.415	0.243	0.283	0.141

Table 13. Pairwise FST for *Icaricia shasta charlestonensis* made using SNPs from MCR50IN nuclear DNA dataset.

	Lee Canyon	**Bonanza**	**South Loop**	**South Sister**
Lee Canyon	--	0.030	0.127	0.037
Bonanza	0.030	--	0.104	0.005
South Loop	0.127	0.104	--	0.106
South Sister	0.037	0.005	0.106	--

Table 14. Genetic parameters reference sheet.

Parameter	**Definition**	***Equation***
Fst	Genetic variation/ structuring in a population.	*1/ (4Nm +1)*
Gis	Inbreeding coefficient. A measure of departure from Hardy-Weinberg proportions within local subpopulations.	*1 − (Ho/Hs)*
H	The number of haplotypes.	-
Ho	Observed heterozygosity.	-
Hs	Heterozygosity within populations.	-
Ht	Total heterozygosity.	-
H't	Corrected total heterozygosity.	-
I	Shannon's Information Index. A measure of the diversity of species in a community.	$I = -\sum pi * ln(pi)$ *p*i: The proportion of the entire community made up of species *i*
m	The number of sequences.	-
n	The number of segregating sites.	-
N	The number of alleles.	-
Na	The average number alleles per SNP locus.	-
Ne	The number of effective alleles.	-

Table 14. Continued.

Parameter	**Definition**	***Equation***
Nm	The number of migrants per breeding season.	(1− Fst)/4Fst
P	The significance level based on 9,999 permutations	-
SS	Sum of Squares	-
ΦST	Analogous to FST but incorporates genealogical relationships among alleles.	
Θ	The rate of drift and loss of genetic diversity.	Θ = 1/2*Ne Θ = ps/a1 Θ = 4Neμ μ: mutation rate
π	Nucleotide diversity. The average number of nucleotide differences per site between any two randomly chosen sequences from a sample population.	$\pi = \sum x_i x_j \pi_{ij}$ x_i *:* the frequency of the *i*th sequence in the population. π_{ij} *:* the number of nucleotide differences per nucleotide site between the *i*th and *j*th sequences.

Figure 1. Big Dune and Lava Dune *Pseudocotalpa giulianii* habitat depicting two subpopulations.

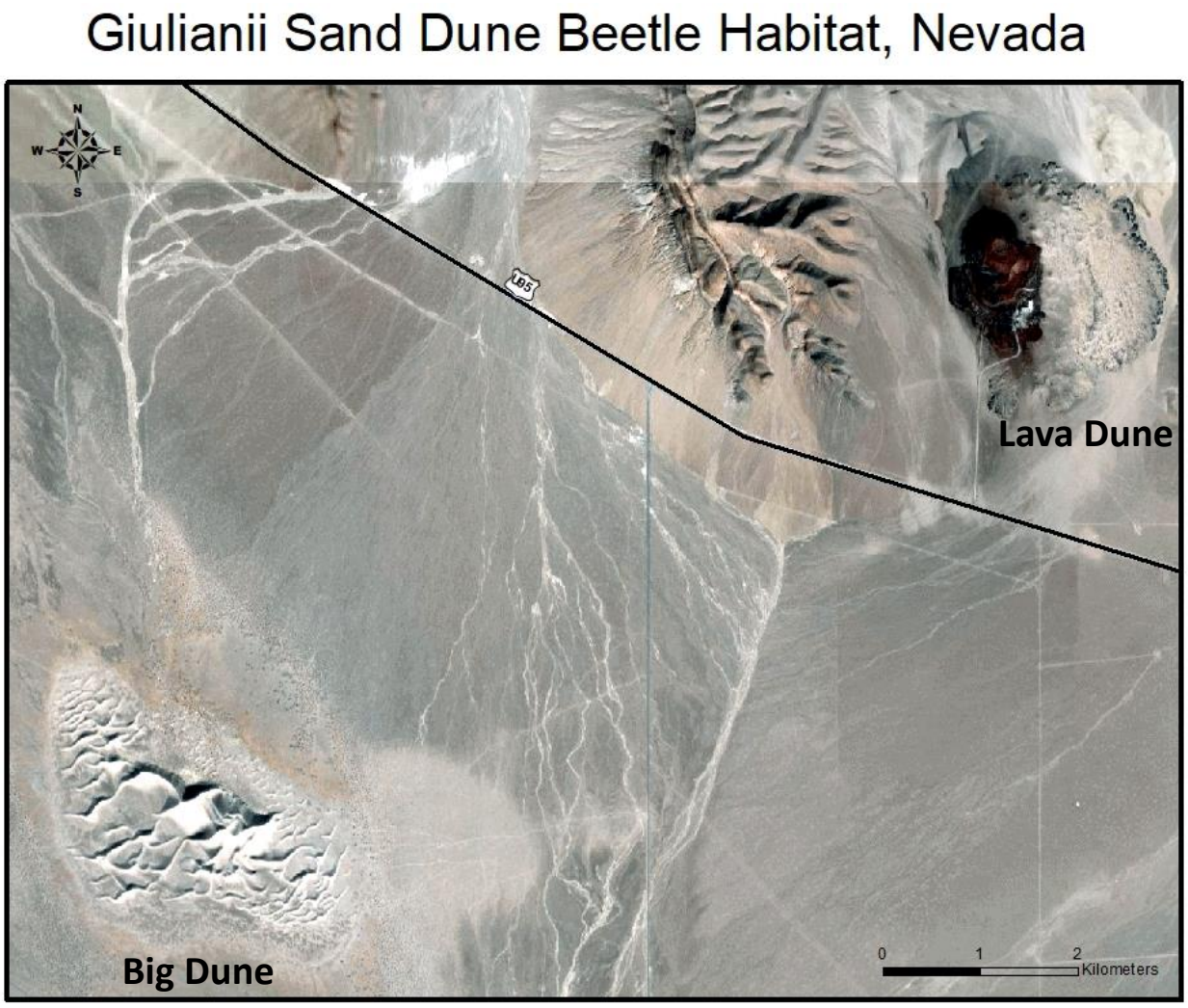


Figure 2. Maximum likelihood tree of *Pseudocotalpa giulianii* mtDNA COI with outgroups. *P. giulianii* collected from Big Dune and Lava Dune, Nevada, one *Cotalpa flavida* from Moapa Nevada, one *Paracotalpa granicolis* from Big Dune and one *Paracotalpa punticolis* from Red Rock Canyon, Nevada. All other outgroup species are from National Center for Biotechnology Information (NCBI). A Le Gascuel model with G and F rate models provided the best fit for the tree (highest log likelihood -5412.25 is shown). The bootstrap consensus tree was inferred from 1000 replicates where the numbers represent the bootstrap support.

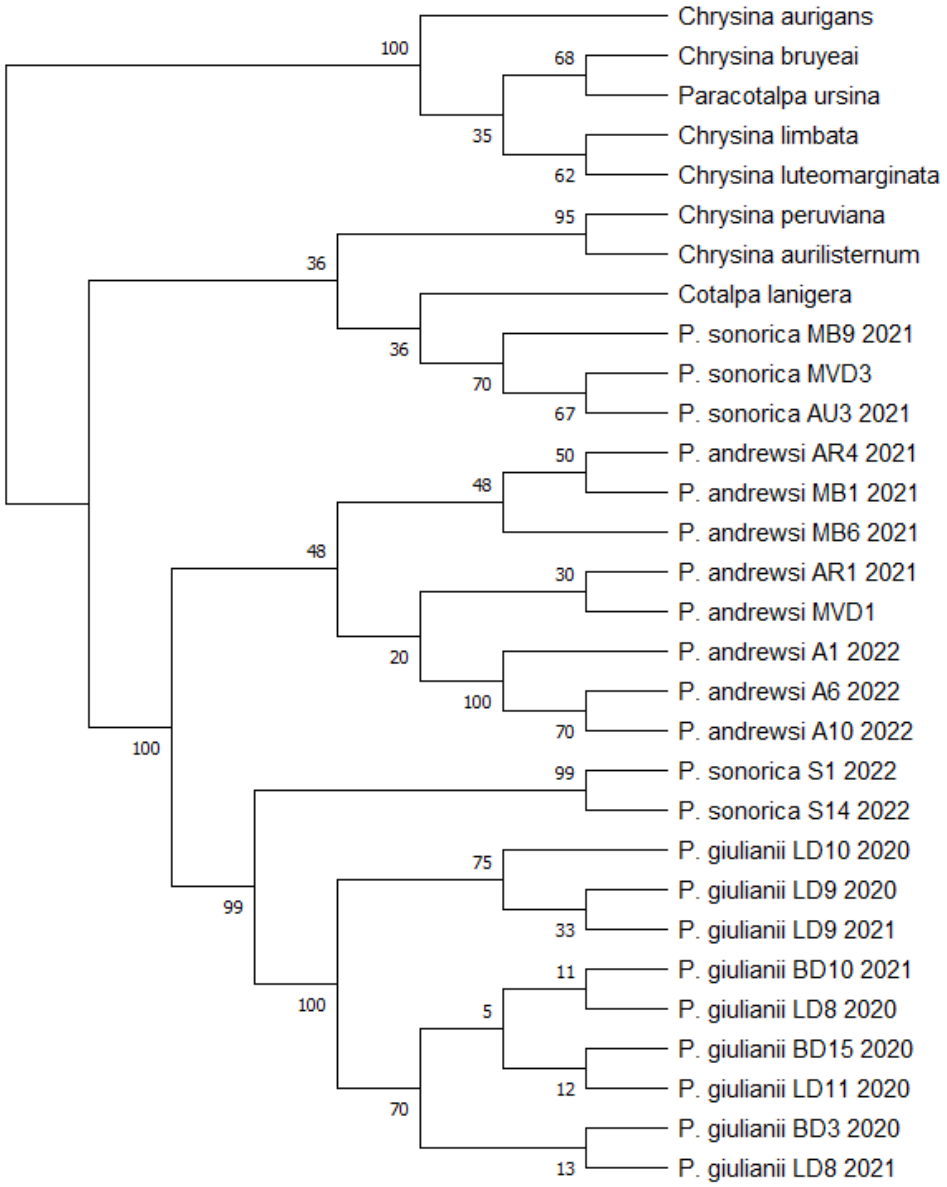


Figure 3. Bayesian inference tree estimated for 18 unique mtDNA COI haplotypes of *Pseudocotalpa* species using *Chrysina, Cotalpa* and *Paracotalpa* species as outgroups. The numbers represent a measure of support for the node.

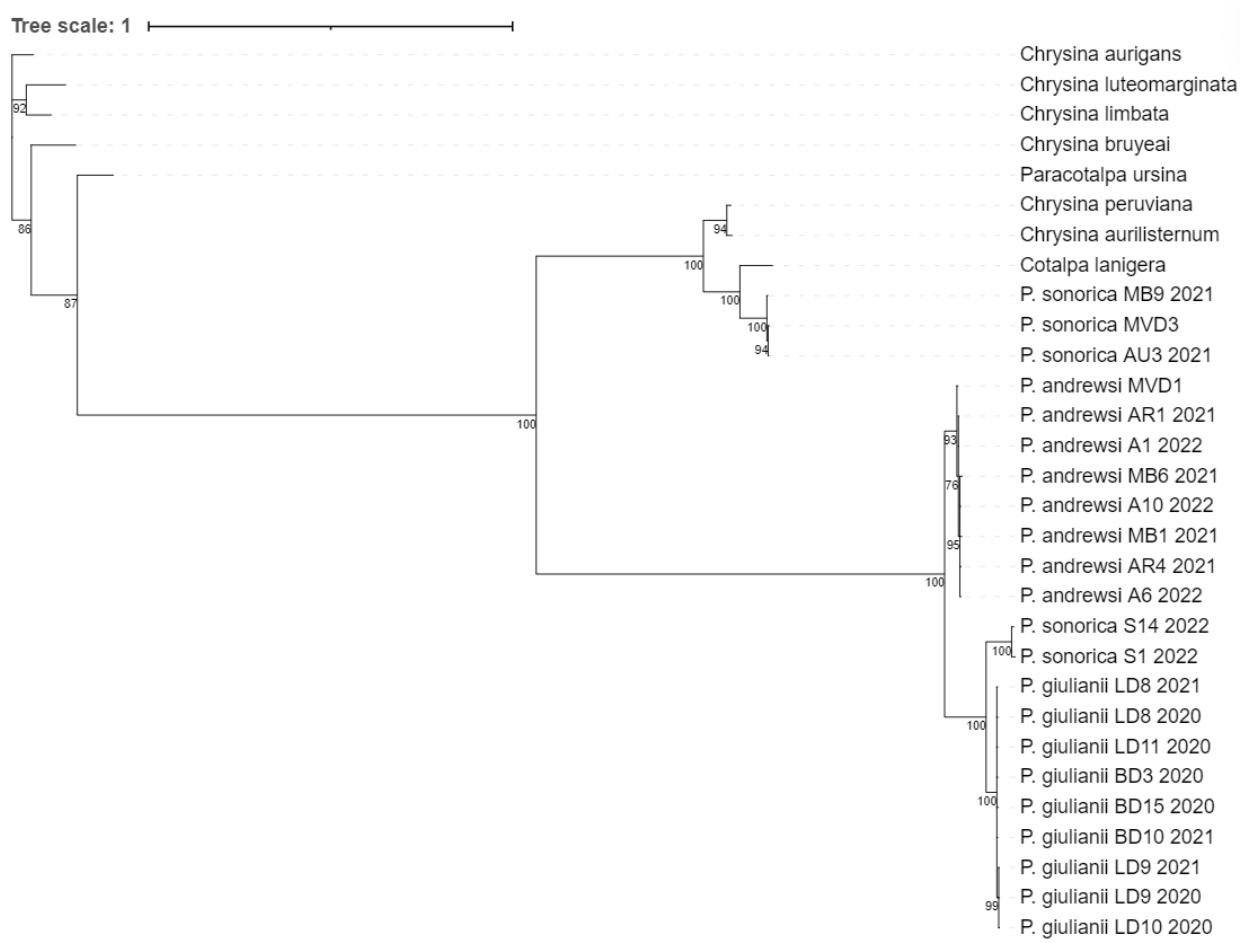


Figure 4. Minimum spanning (TCS) network of four *Pseudocotalpa giulianii* mtDNA COI haplotypes from the Amargosa Valley, Nevada. The circle size reflects the number of individuals exhibiting a haplotype and the hatch marks represent the number of nucleotide differences between the 594 base pairs.

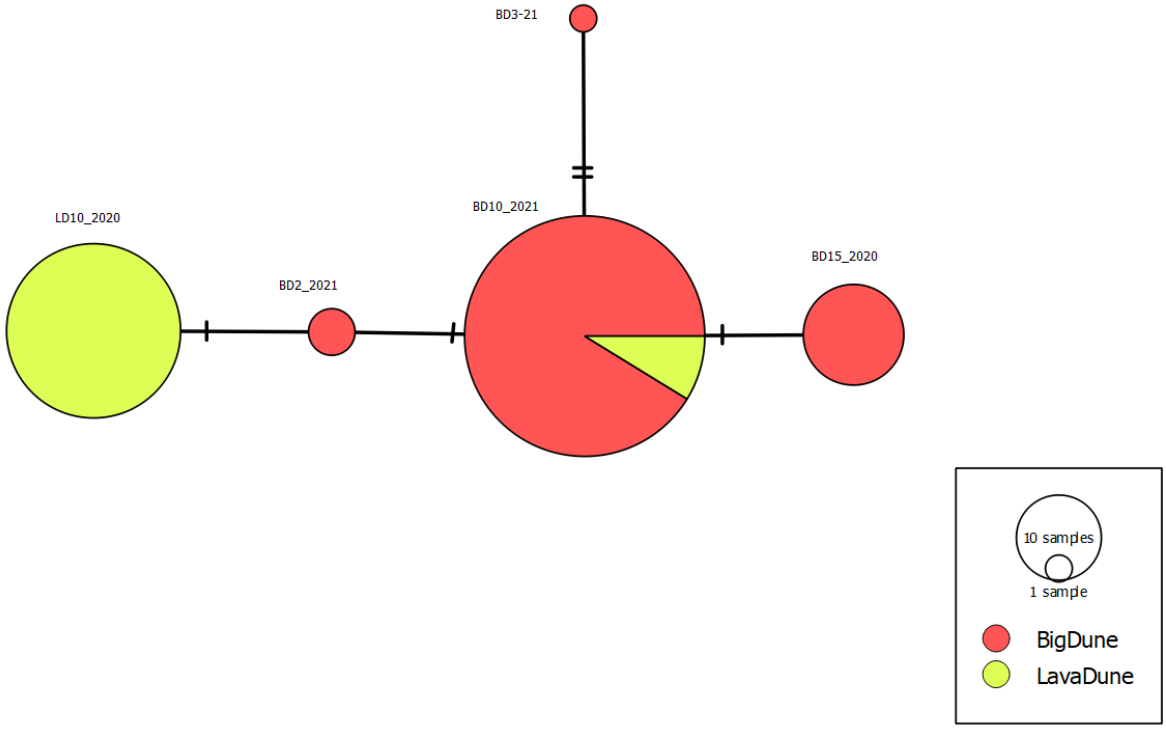


Figure 5. *Pseudocotalpa* phylogenetic tree made using SNPs nuclear DNA dataset. Refer to Table 2 for species labeling.

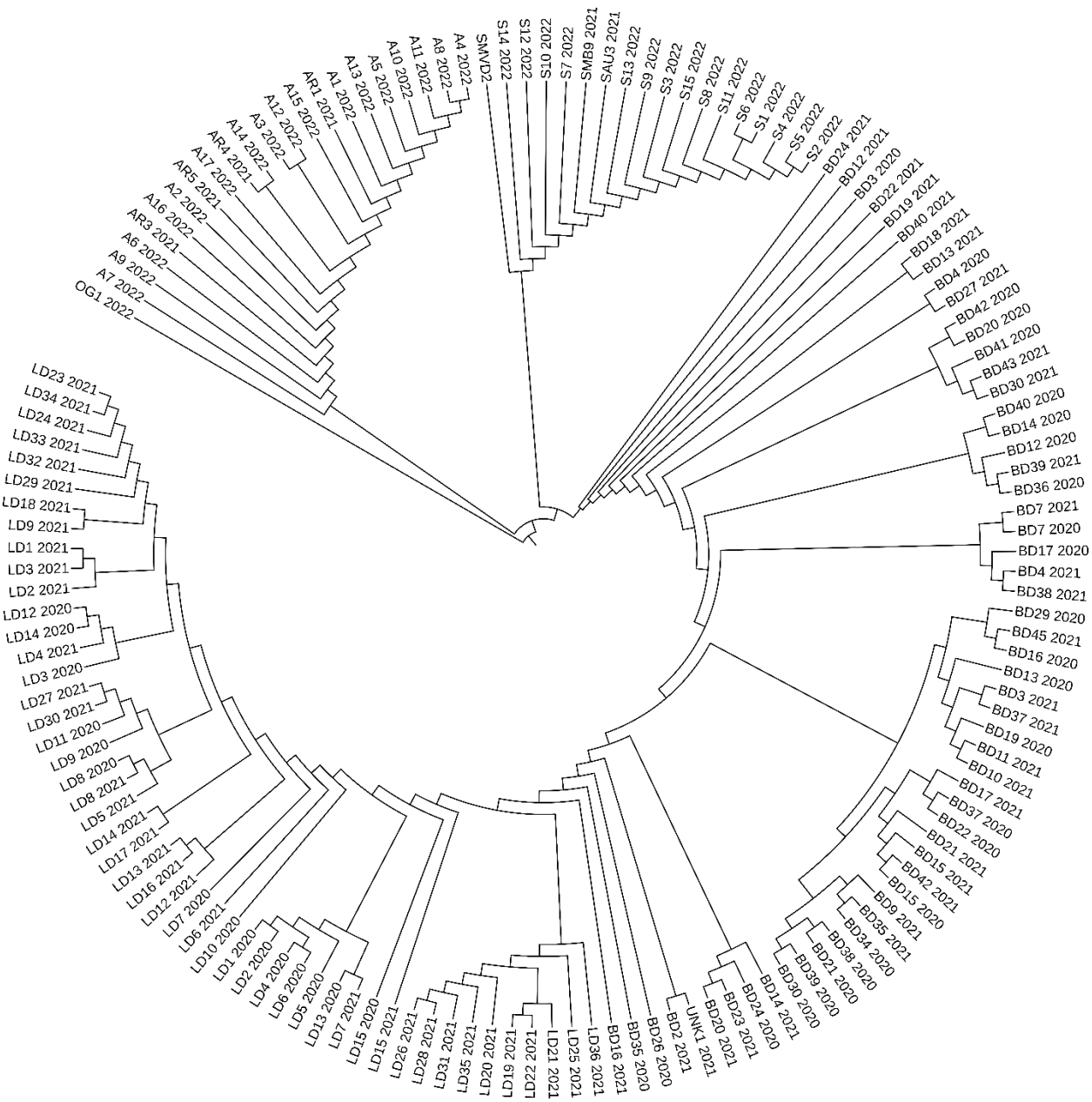


Figure 6. Spring mountain *Icaricia shasta charlestonensis* habitat depicting 4 subpopulations. Blue: Bonanza Trail, Green: South Sister Trail, Purple: Lee Canyon Ski Area, Orange: South Loop Trail.

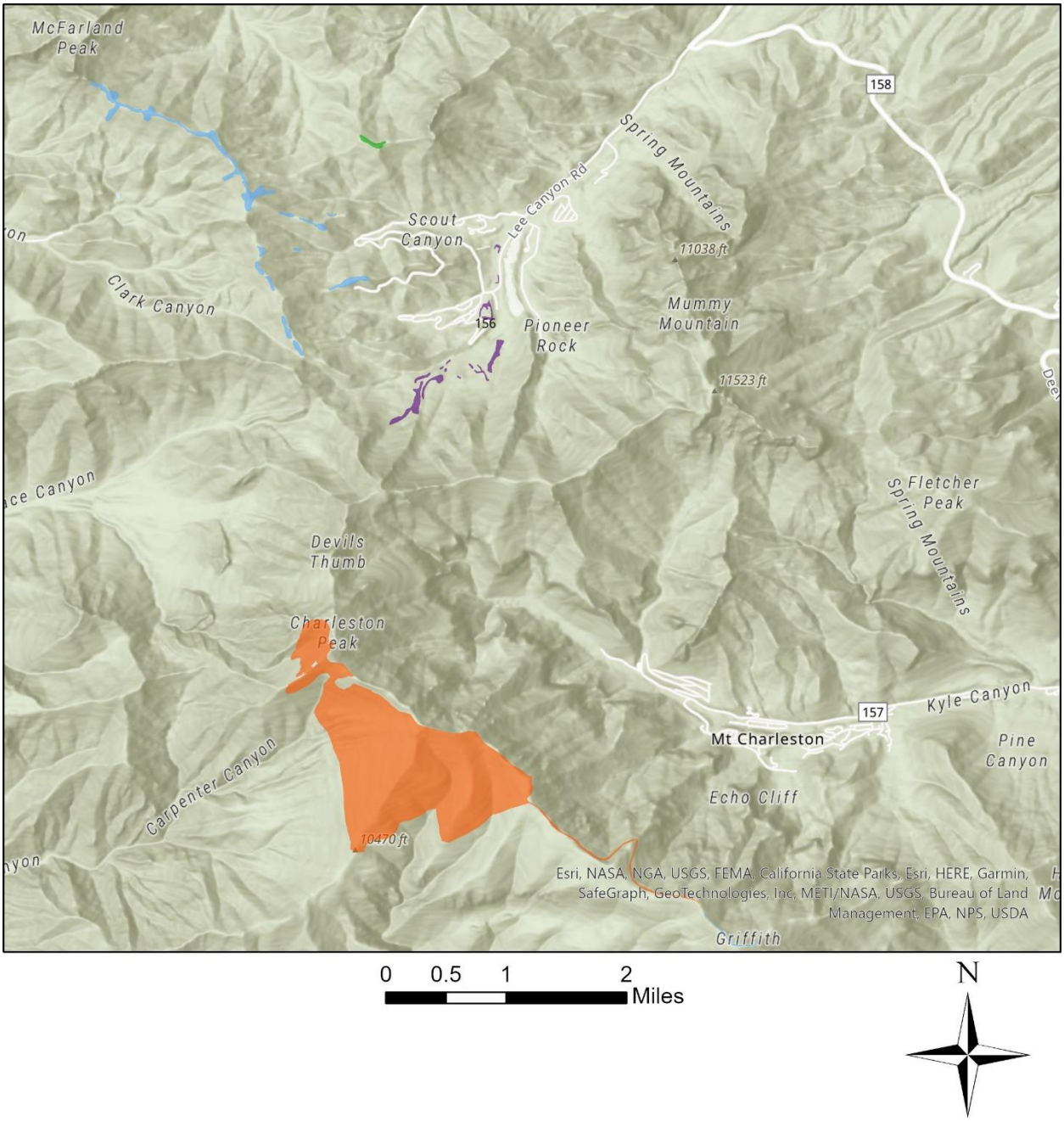


Figure 7. Maximum likelihood tree of eight *Icaricia shasta charlestonensis* and outgroup species *Icaricia shasta minnehaha, Euphilotes ancilla purpura* and *Icaricia icarioides austinorum* mtDNA COI haplotypes. A Tamura-Nei model with G and I rate models provided the best fit for the tree (highest log likelihood -2892.81 is shown). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed where the numbers represent the bootstrap support.

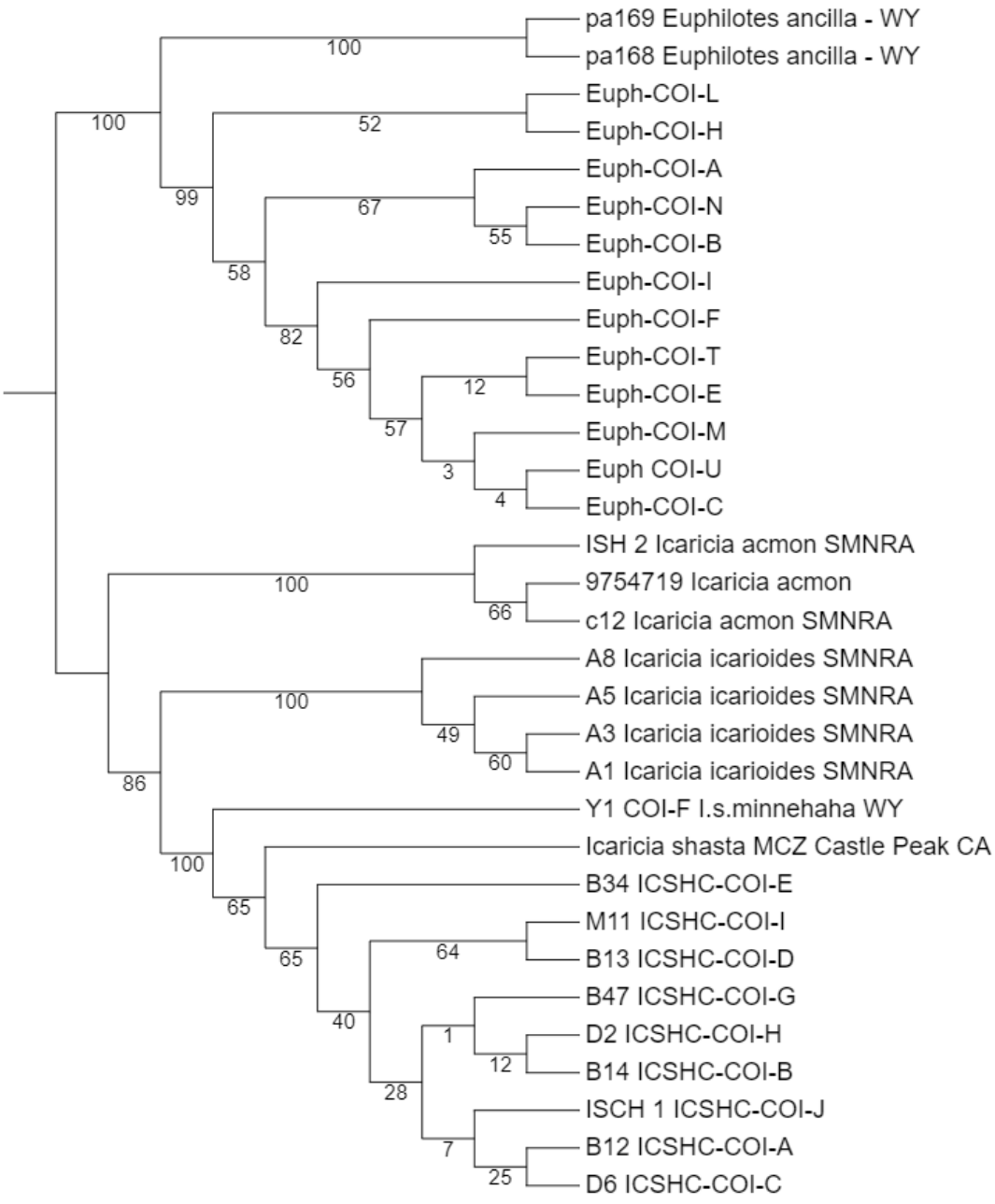


Figure 8. Minimum spanning network of eight *Icaricia shasta charlestonensis* and one *Icaricia shasta minnehaha* mtDNA COI haplotypes. The circle size reflects the number of individuals exhibiting a haplotype and the hatch marks represent the number of nucleotide differences between the 1224 base pairs.

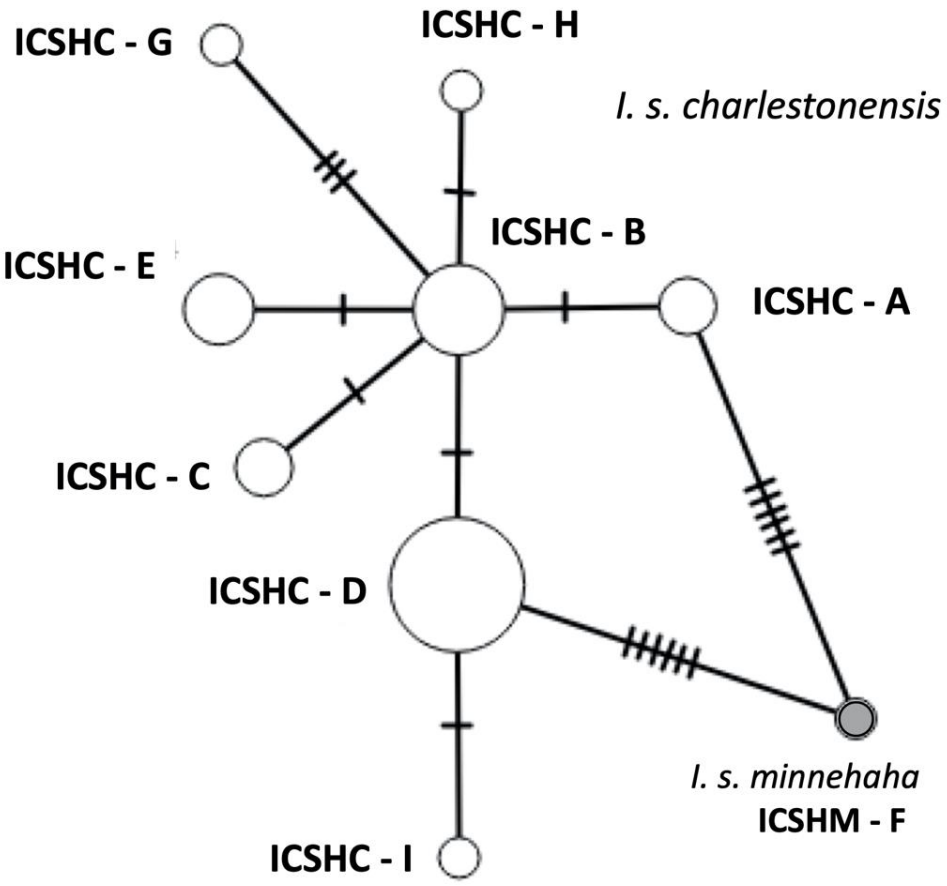


Figure 9. Genetic clustering of 101,253 SNP loci (MCR50IN) estimated by STRUCTURE for *Icaricia shasta charlestonensis.* The 3 to 4 genetically distinct groups are color coded (purple, blue, orange, green). Each vertical bar represents one individual, each cluster is represented by one color. The locations individuals were collected are Lee Canyon ski area, South Sister, South Loop Trail, and Bonanza Trail.

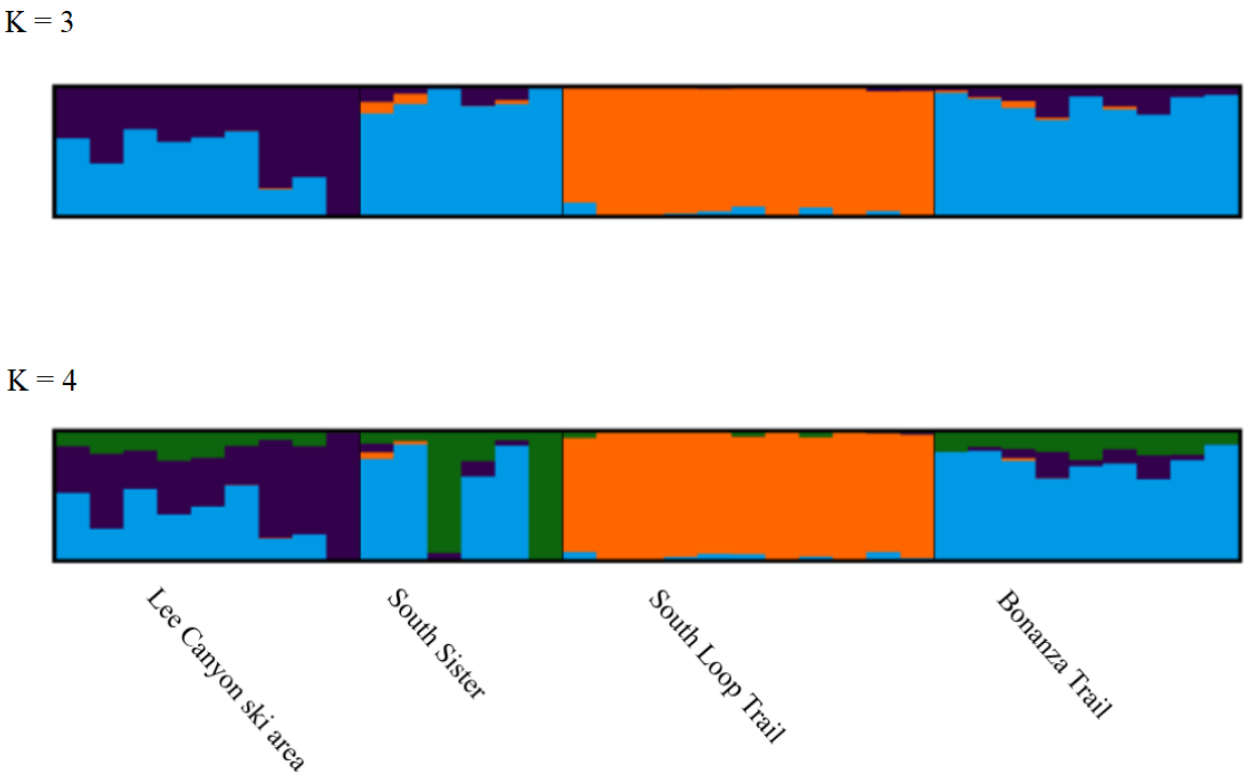


Figure 10. Principle Coordinate Analysis of *Icaricia shasta charlestonensis* made using 8190 SNPs from nuclear DNA dataset.

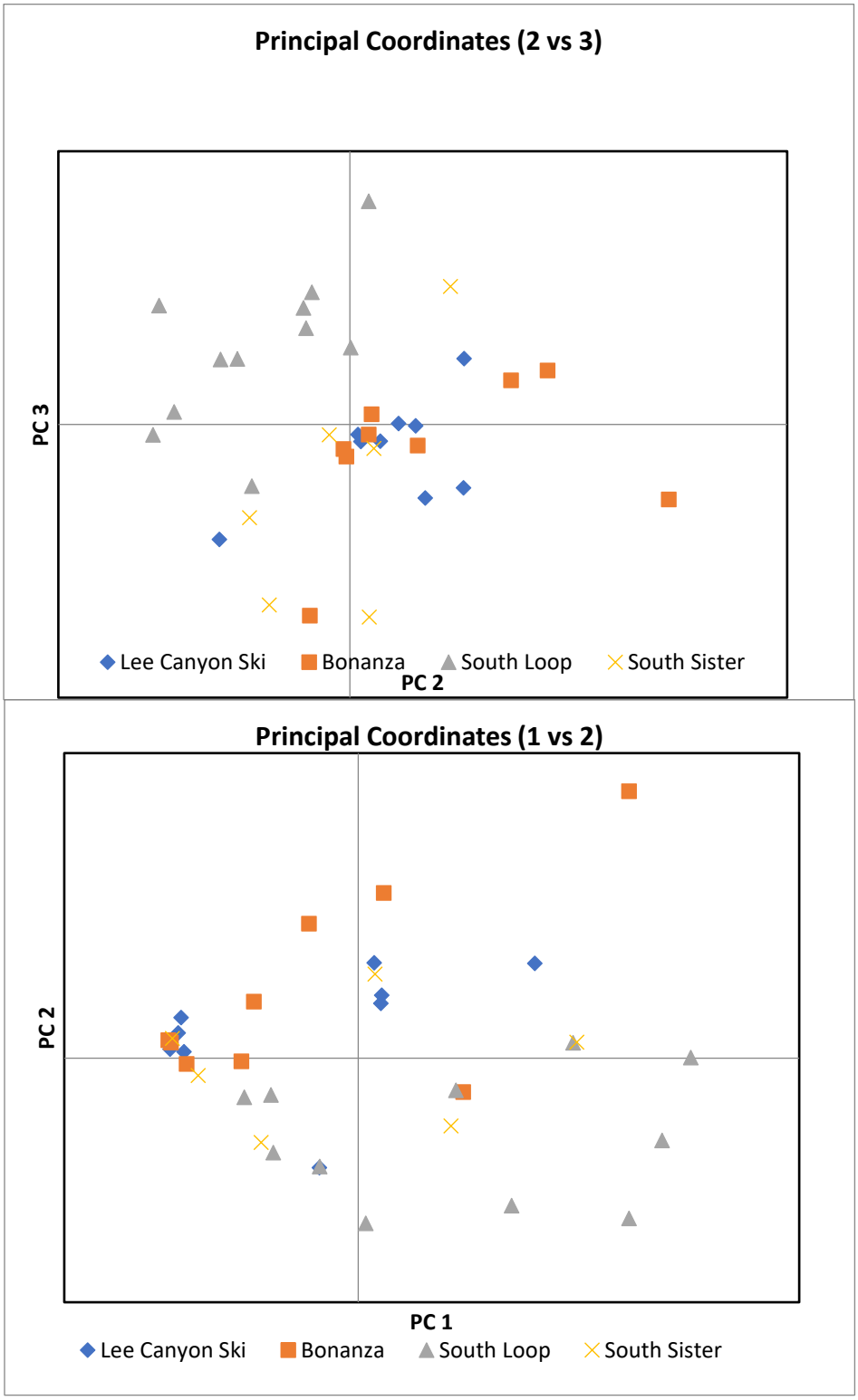
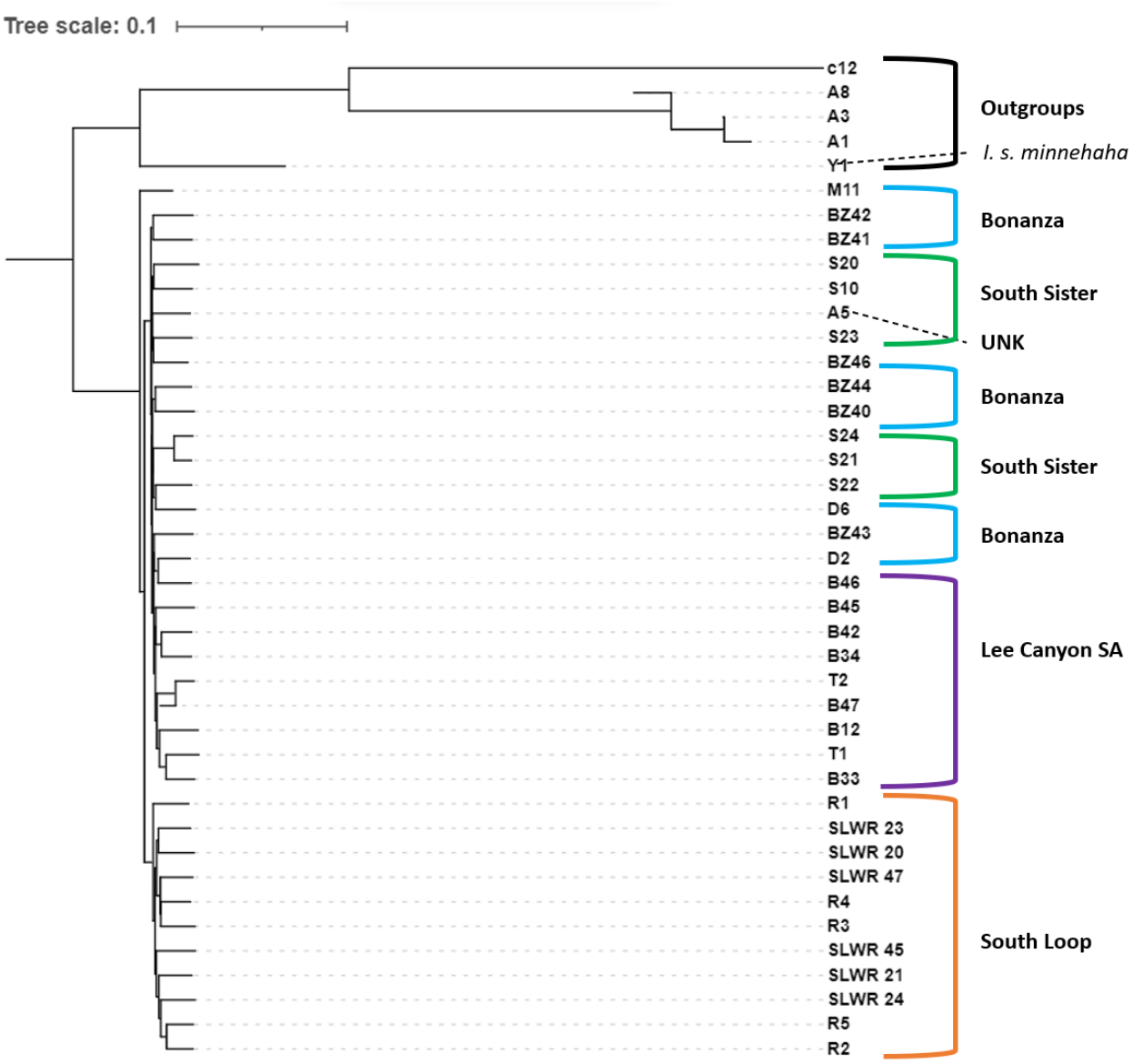


Figure 11. Phylogenetic tree of *Icaricia shasta charlestonensis* using the MCR50 SNP nuclear DNA dataset. Outgroup species are Y1: *Icaricia shasta minehaha*, c12: *Plebejus acmon*, A8, A3, A1: *Icaricia icarioides austinorum*, A5: Unknown location *Icaricia shasta charlestonensis*.



References

Allendorf, F. W., Luikart, G. H., Aitken, S. N., & Aitken, S. N. (2012). Conservation and the genetics of populations. ProQuest Ebook Central

Allendorf F.W. (2017) Genetics and the conservation of natural populations: allozymes to genomes. Mol Ecol 26:420–430

Andrews, F. G., Hardy, A. R., & Giuliani, D. (1979). The Coleopterous Fauna of Selected California Sand Dunes: A Report. Insect Taxonomy Laboratory, Division of Plant Industry, California Department of Food and Agriculture.

Andrew G, Glenn M, Jacoby-Garrett P, Thompson DB. (2013). Surveys and Habitat

Assessment for *Plebejus shasta charlestonensis* (Mount Charleston Blue Butterfly) in the Spring Mountain Range of Nevada (2012 field season). March 6, 2013. Final Revision December 11 (w/new Figures). Las Vegas, Nevada: Submitted to United States Fish and Wildlife Service, Southern Nevada Field Office, 94 pp.

Brown, J. H. (1984). On the relationship between abundance and distribution of species. The american naturalist, 124(2), 255-279.

California Natural Diversity Database (October 2021). Special Animals List. California Department of Fish and Wildlife. Sacramento, CA.

Chan, L. M., Painter, C. W., Hill, M. T., Hibbitts, T. J., Leavitt, D. J., Ryberg, W. A., Walkup, D., & Fitzgerald, L. A. (2020). Phylogeographic structure of the dunes sagebrush lizard, an endemic habitat specialist. PLOS ONE, 15(9), e0238194.

Crawford, L. A., Desjardins, S., & Keyghobadi, N. (2011). Fine-scale genetic structure of an endangered population of the Mormon metalmark butterfly (*Apodemia mormo*) revealed using AFLPs. Conservation Genetics, 12(4), 991–1001.

Dennis, R. L. H. (2004). Just how important are structural elements as habitat components? Indications from a declining lycaenid butterfly with priority conservation status. Journal of Insect Conservation, 8(1), 37–45.

Dennis, R. L. H., Dapporto, L., & Dover, J. W. (2014). Ten years of the resource-based habitat paradigm: The biotope-habitat issue and implications for conserving butterfly diversity. Journal of Insect Biodiversity, 2(8), 1.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., ... & Gascuel, O. (2008). Phylogeny. fr: robust phylogenetic analysis for the non-specialist. Nucleic acids research, 36(suppl_2), W465-W469.

Dereeper A., Audic S., Claverie J.M., Blanc G. (2010). BLAST-EXPLORER helps you building datasets for phylogenetic analysis. BMC Evol Biol. Jan 12;10:8. (PubMed)

Dunn, R. R. (2005). Modern insect extinctions, the neglected majority. Conservation biology, 19(4), 1030-1036.

Dupuis, J. R., Geib, S. M., Osborne, K. H., & Rubinoff, D. (2020). Genomics confirms surprising ecological divergence and isolation in an endangered butterfly. Biodiversity and Conservation, 29(6), 1897–1921.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14:2611–2620.

Falush D, Stephens M, Pritchard JK (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164:1567–1587

Folmer, O., Hoeh, W. R., Black, M. B., & Vrijenhoek, R. C. (1994). Conserved primers for PCR amplification of mitochondrial DNA from different invertebrate phyla. Molecular Marine Biology and Biotechnology, 3(5), 294-299.

Frankham R, Ballou JD, Briscoe DA (2002) 'Introduction to Conservation Genetics'. (Cambridge University Press: Cambridge.)

Franklin, I. R. (1980). Evolutionary change in small populations.

Fu Y. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147: 915–925.

Funk, W. C., McKay, J. K., Hohenlohe, P. A., & Allendorf, F. W. (2012). Harnessing genomics for delineating conservation units. Trends in Ecology & Evolution, 27(9), 489–496.

Hanski, I., & Kuussaari, M. (1995). Butterfly metapopulation dynamics. Population dynamics: new approaches and synthesis, 8, 149-171.

Hardy, A. R. (1974). Two new species of Pseudocotalpa Hardy (Coleoptera: Scarabaeidae). Panpac Entomol.

Hardy, A. R. (1976). Observations on the mating behavior of *Pseudocotalpa giulianii*. Coleopterists Bulletin 30(3): 301-302.

Hobolth, A., Dutheil, J. Y., Hawks, J., Schierup, M. H., & Mailund, T. (2011). Incomplete lineage sorting patterns among human, chimpanzee, and orangutan suggest recent orangutan speciation and widespread selection. Genome research, 21(3), 349-356.

Huelsenbeck JP., Ronquist F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics., Aug;17(8):754-5. (PubMed)

Hunt, T., Bergsten, J., Levkanicova, Z., Papadopoulou, A., John, O. St., Wild, R., Hammond, P. M., Ahrens, D., Balke, M., Caterino, M. S., Gomez-Zurita, J., Ribera, I., Barraclough, T. G., Bocakova, M., Bocak, L., & Vogler, A. P. (2007). A Comprehensive Phylogeny of Beetles Reveals the Evolutionary Origins of a Superradiation. Science, 318(5858), 1913–1916.

Jameson, M. L. (n.d.). Phylogenetic Analysis of the Subtribe Rutelina and Revision of the Rutela Generic Groups (Coleoptera: Scarabaeidae: Rutelinae: Rutelini). 192.

Jeppsson, T., & Forslund, P. (2014). Species' traits explain differences in Red list status and long-term population trends in longhorn beetles. Animal Conservation, 17(4), 332-341.

Keller I, Largiader CR (2003). Recent habitat fragmentation caused by major roads leads to reduction of gene flow and loss of genetic variability in ground beetles. Proc R Soc Lond B 270:417–423

Kutschera, V. E., Bidon, T., Hailer, F., Rodi, J. L., Fain, S. R., & Janke, A. (2014). Bears in a Forest of Gene Trees: Phylogenetic Inference Is Complicated by Incomplete Lineage Sorting and Gene Flow. Molecular Biology and Evolution, 31(8), 2004–2017.

Kopelman NM, Mayzel J, Jakobsson M, Rosenberg NA, Mayrose I (2015. Clumpak: a program for identifying clustering modes and packaging population structure inferences across K. Mol Ecol Resour 15:1179–1191. https://doi.org/10.1111/1755-0998.12387

Lausen, C. L., Delisle, I., Barclay, R. M. R., & Strobeck, C. (2008). Beyond mtDNA: nuclear gene flow suggests taxonomic oversplitting in the little brown bat (Myotis lucifugus). Canadian Journal of Zoology, 86(7), 700-713.

Leigh, J. W., & Bryant, D. (2015). POPART: full-feature software for haplotype network construction. Methods in Ecology and Evolution, 6(9), 1110-1116.

Lester, S. E., Ruttenberg, B. I., Gaines, S. D., & Kinlan, B. P. (2007). The relationship between dispersal ability and geographic range size. Ecology Letters, 10(8), 745-758.

Lowe, W.H. & Allendorf, F.W. (2010). What can genetics tell us about population connectivity? Molecular Ecology, 19, 3038–3051.

Luckenbach, R. A., and R. B. Bury. (1983). Effects of off-road vehicles on the biota of the Algodones Dunes, Imperial County, California. J. Appl. Ecol. 20: 265Ð286.

Mallet, J. (2005). Hybridization as an invasion of the genome. Trends in Ecology & Evolution, 20(5), 229-237.

Marko, P. B., & Hart, M. W. (2011). The complex analytical landscape of gene flow inference. Trends in Ecology & Evolution, 26(9), 448–456.

Min, H.-K., Kim, D. S., & Cho, Y.-B. (2018). The monitoring of sand dune insects using pitfall trap in Goraebul beach, Yeongdeok-gun, South Korea. Journal of Asia-Pacific Biodiversity, 11(1), 32–38. https://doi.org/10.1016/j.japb.2017.11.001

Muñoz, P. T., Torres, F. P., & Megías, A. G. (2015). Effects of roads on insects: a review. Biodiversity and Conservation, 24(3), 659-682.

Nathan, L. R., Kanno, Y., & Vokoun, J. C. (2017). Population demographics influence genetic responses to fragmentation: A demogenetic assessment of the 'one migrant per generation' rule of thumb. Biological Conservation, 210, 261–272.

Nei, M. (1987). Molecular Evolutionary Genetics. Columbia University Press, New York.

Ott, A., Liu, S., Schnable, J. C., Yeh, C.-T. 'Eddy,' Wang, K.-S., & Schnable, P. S. (2017). TGBS® genotyping-by-sequencing enables reliable genotyping of heterozygous loci. Nucleic Acids Research, 45(21), e178.

Nolte, D., Boutaud, E., Kotze, D. J., Schuldt, A., & Assmann, T. (2019). Habitat specialization, distribution range size and body size drive extinction risk in carabid beetles. Biodiversity and Conservation, 28(5), 1267–1283.

Pritchard JK, Stephens M., Donnelly P. (2000) Inference of population structure using multilocus genotype data. Genetics, 155, 945–959.

Pavlik, B. M. (1989). Phytogeography of sand dunes in the Great Basin and Mojave Deserts. Journal of Biogeography, 227-238.

Peakall, R. O. D., & Smouse, P. E. (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular ecology notes, 6(1), 288-295.

Ramírez-Soriano A, Ramos-Onsins SE, Rozas J, Calafell F, Navarro A. (2008). Statistical power analysis of neutrality tests under demographic expansions, contractions and bottlenecks with recombination. Genetics, 179:555–567

Ritcher, P. O. (1958). Biology of scarabaeidae.

Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado, P, Ramos-Onsins SE, Sánchez-Gracia A. (2017). DnaSP 6: DNA Sequence Polymorphism Analysis of Large Datasets. Mol. Biol. Evol. 34: 3299-3302.

Schebeck, M., Hansen, E. M., Schopf, A., Ragland, G. J., Stauffer, C., & Bentz, B. J. (2017). Diapause and overwintering of two spruce bark beetle species. Physiological Entomology, 42(3), 200-210.

Schultz, C. B. (1998). Dispersal Behavior and Its Implications for Reserve Design in a Rare Oregon Butterfly. Conservation Biology, 12(2), 284–292.

Sever, A. (2011). Butterfly Monitoring and Inventories Spring Mountains National Recreation Area. Clark County.

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD, Higgins DG. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7:539

Slatkin, M. (1987). Gene Flow and the Geographic Structure of Natural Populations. Science, 236(4803), 787–792.

Sol, D. (2008). Do successful invaders exist? Pre-adaptations to novel environments in terrestrial vertebrates. In Biological invasions (pp. 127-141). Springer, Berlin, Heidelberg.

Stockwell, C. A., Hendry, A. P., & Kinnison, M. T. (2003). Contemporary evolution meets conservation biology. Trends in Ecology & Evolution, 18(2), 94–101.

Tajima F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123: 585–595.

Thompson D B. 2017. Report on Mount Charleston Blue Butterfly Research in SMNRA under FWS Permit 10(A)(1)(a)-TE63440BL0. Unpublished report to the U.S. Fish and Wildlife Service, Southern Nevada Office, Las Vegas, Nevada.

Thompson DB. 2018. Surveys and Habitat Assessment of *Icaricia shasta charlestonensis* (Mount Charleston Blue Butterfly) in the Spring Mountain Range of Nevada, 2015-2017, Final Report. Unpublished report to the U.S. Fish and Wildlife Service, Southern Nevada Office, Las Vegas, Nevada. 10 October 2018.

Thompson DB. 2022. Mount Charleston Blue Butterfly Population and Habitat Status Surveys 2019-2021 (F19AC00089 - 01). Unpublished report to the U.S. Fish and Wildlife Service, Southern Nevada Office, Las Vegas, Nevada. 13 May 2022

Tscharntke, T., Steffan-Dewenter, I., Kruess, A., & Thies, C. (2002). Characteristics of insect populations on habitat fragments: A mini review: Insect populations and habitat fragments. Ecological Research, 17(2), 229–239.

Ugelvig LV, Vila R, Pierce NE, Nash DR (2011) A phylogenetic revision of the Glaucopsyche section (Lepidoptera: Lycaenidae), with special focus on the *Phengaris–Maculinea* clade. Mol Phylogenet Evol 61:237–243

Van Dam, A. R., & Van Dam, M. H. (2008). Impact of Off-Road Vehicle Use on Dune Endemic Coleoptera. Annals of the Entomological Society of America, 101(2), 411–417.

Weiss, S. B., Weiss, A. D., Murphy, D. D., & Austin, G. T. (1997). Final report on endemic butterflies of the Spring Mountains. Unpublished report prepared for US Fish and Wildlife Service, Reno, NV.

Wiggs, G.F.S., D.S.G. Thomas, J. E. Bullard, and I. Livingstone. (1995). Dune mobility and vegetation cover in the southwest Kalahari Desert. Earth Surf. Process. Landf. 20: 515Ð529.

Wilson JS, Sneck M, Murphy DD, Nice CC, Fordyce JA, Forister ML (2013). Complex evolutionary history of the pallid dotted-blue butterfly (Lycaenidae: Euphilotes pallescens) in the Great Basin of western North America. J Biogeogr 40:2059–2070

Wright, S. (1969). The theory of gene frequencies. Evolution and the Genetics of Populations, 2, 290-344.

Wright, S. (1978). Evolution and the genetics of populations. Volume 4. Variability within and among natural populations. University of Chicago Press, Chicago.

Wu, T. and Nacu, S. (2010). Fast and snp-tolerant detection of complex variants and splicing in short reads. Bioinformatics, 26(7):873–881.

Zhang, S.-Q., Che, L.-H., Li, Y., Dan Liang, Pang, H., Ślipiński, A., & Zhang, P. (2018). Evolutionary history of Coleoptera revealed by extensive sampling of genes and species. Nature Communications, 9(1), 205. https://doi.org/10.1038/s41467-017-02644-4

Curriculum Vitae

Kristen Tovar

Personal Information

Email: kristentovar@gmail.com

Education

University Of Hawaii at Manoa, Honolulu, HI

Bachelor of Science in Marine Biology, Spring 2015

University of Nevada Las Vegas, Las Vegas, NV.

Master of Science in Biology (Ecology and Evolution): Fall 2020 – Spring 2023 (Research Advisor: Dr. Daniel Thompson)

Professional Experience

8/2022 – 7/2023	Lab instructor, Biology 191 – Introduction to Biology University of Nevada, Las Vegas
1/2020 – 8/2020	Avian Biologist Western EcoSystems Technology
8/2019 – 1/2020	Landscape Supervisor Tovar Landscape and Design Inc.
10/2019 – 4/2020	Consulting Field Biologist Newfields
4/2019 – 7/2019	Avian Biologist/ Surveyor

	Turnstone Environmental Consultants
12/2018 – 3/2019	Park Ranger Department of Conservation
9/2015 – 4/2018	Field Technician The Research Corporation of the University of Hawaii
4/2017 – 8/2017	Hawaiian Monk Seal Research Program Field Camp Volunteer NOAA
7/2015 – 3/2017	Hawaiian Monk Seal Project Volunteer NOAA
8/2014 – 9/2015	Monk Seal Trainer Internship Waikiki Aquarium
11/2011 – 4/2012	Resident Assistant University of Hawaii at Manoa
8/2011 – 05/2015	Student Minister Newman Center at the University of Hawaii
9/2010 – 12/2013	Shark Research Internship Hawaii Institute of Marine Biology at Coconut Island