University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
Fall 2023

Patterns of genetic diversity in a large, isolated population of American Ginseng (*Panax quinquefolius* L) in western North Carolina

Anna Joel

Biology Department
The University of North Carolina Asheville
One University Heights
Asheville, North Carolina 28804 USA

Faculty Mentor: Dr. Jennifer Rhode Ward

Abstract

American Ginseng (*Panax quinquefolius* L) is a long-lived understory plant in the Araliaceae family. The genus *Panax* has been used in eastern medicine for thousands of years and has chemical and morphological properties that make collecting wild *P. quinquefolius* very profitable. Overharvesting, habitat loss, and herbivory can decrease genetic diversity, devastating populations of American Ginseng. Genetic testing was performed on leaflet samples (N = 163) collected from a large, robust population in Buncombe County, NC, which was hypothesized to be more diverse than others in the region because of its size. DNA was extracted from the samples and was PCR amplified for further testing of seven microsatellite loci. Data were compared to values from 11 other regional populations using the *polysat* package in R; differences among populations were measured using principal component analysis, and allelic and genotypic frequencies were also estimated. The principal component analysis showed that the focal population was genetically distinct from other populations and had high

levels of interpopulation diversity. This population also had the highest allelic and genotypic diversity of the 12 tested, supporting the initial hypothesis. Genetic diversit was also compared in morphological groups of LK. Reproductive plants showed higher genetic diversity than non reproductive plants perhaps due to a link between genetic diversity and population fitness. Larger plants were higher in genetic diversity than small plants as seen in other studies of unprotected populations of American Ginseng. The high genetic diversity of this population makes it a conservation priority. It might also be useful as a source of seeds for restoration or augmentation.

Introduction

Small, Isolated populations are of conservation concern because they are more susceptible to the loss of genetic diversity due to ecological and environmental disturbances. In isolated plant populations, genetic diversity can be positively linked with population size (Lammi et al, 1999). A meta analysis by Reed and Frankham in 2003, found that genetic diversity and population size are positively correlated with population fitness. The genetic diversity of a population is important to assess along with demographic information to make conservation decisions (Leimu et al. 2006). For instance, a study of native Brazilian trees showed that genetic testing combined with demographic data gave a more accurate assessment of conservation status than demographic data alone (Muniz, 2019). Sarrou (2022), working with a perennial medicinal plant, used a combination of genetic and demographic data to prioritize the least diverse population for conservation efforts. Population fitness can be measured by characteristics of reproduction such as seed production, germination ability, number of flowers, and fruit growth (Ilves et al. 2013), (Leimu et al. 2006). Population fitness is helpful in determining a populations persistence and ability to adapt to changing environments. American Ginseng blooms hermaphroditic flowers and experiences a mixed mating system. According to a review published in 1984 by Loveless and Hamrick, they predicted these characteristics correlate with moderate to low genetic diversity with in a population (Loveless and Hamrick, 1984). American Ginseng often grows in isolated or fragmented populations, and genetic diversity in wild populations might be low compared to cultivated populations (Grubbs and Case, 2004). The anthropogenic processes on wild American Ginseng populations make it essential that we clearly understand factors that can inform conservation decisions.

American Ginseng (*Panax quinquefolius* L) is a long living herb in the Araliaceae family that can grow over half a meter tall. *Panax quinquefolius* grows in the understory of deciduous forests in central and eastern North America from Georgia to Quebec,

Canada (USDA, 2023). American Ginseng's native range is vast but populations are small, usually consisting of less than 200 individuals and isolated from one another (McGraw et al, 2013). It experiences a slow life cycle taking several years to reach maturity. Young individuals have one or two leaves. Mature specimens feature 3 to 6 leaves, each of which typically has five leaflets. Ginseng is a perennial, the above ground becomes dormant in the winter and comes back the following year. Depending on environmental stress, mature Ginseng can come back as a 2 leaf plant, so it can sometimes be difficult to properly determine the age based on the number of leaves. Ginseng has a mixed mating system that includes outcrossing and self fertilizing. Reproductive plants produce many whitish-greenish hermaphroditic flowers that get pollinated by bees and flies. Flowers bloom on the central stem in summer and produce bright red berries in the fall. Berries are dispersed by birds such as Wood Thrush (*Hylocichla mustelina*)(USDA, 2023)(McGraw et al. 2022).

American Ginseng has immense cultural and medicinal value. The root of Ginseng is the most valuable in the global market, and it increases in value with the age of the plant. The genus *Panax* has been in use for its medicinal properties in east Asian and north american indigenous cultures for thousands of years (McGraw et al. 2013). Ginseng continues to be used for anti-inflammation, immune support, anticancer, and cardiovascular supporting properties (Wee, 2011). These properties come from a major group of bioactive compounds found in the root of Ginseng, saponins known as ginsenosides. The variety of ginsenoside compounds might be related to Ginseng's multiple medicinal effects on human diseases. Other non-saponin components produced by the plant that have important human health properties include essential oils, peptides, amino acids, antioxidants, polysaccharides, and vitamins (Wee, 2011). The Ginseng market, based in Traditional Asian Medicine, values wild Ginseng because wild Ginseng root is more gnarled and appears humanoid. Cultivated Ginseng root is more straight and tubular (Grubbs and Case, 2004). These root properties make wild *P. quinquefolius* very profitable and susceptible to overharvesting on the landscape (Anderson et al. 2002).

One of the biggest threats to American Ginseng populations is overharvesting for export. This has been happening since the 1700s and prompted CITES to add it to the its endangered species list in 1975 (McGraw et al. 2013). Regulations in the United States and in Canada were put in place after that but are challenging to enforce(McGraw et al. 2013). In a statement made in 2021, the National Forest Service restricted the harvesting of American Ginseng on park land in the Pisgah and Nantahala National Forests, after many failed attempts to enforce restrictions on harvesting in specific seasons and instances of irresponsible harvesting (Forest Service, 2021). Overharvesting negatively affects individual populations on a genetic and demographic

level and, acting as genetic bottlenecks causing loss of potential beneficial genetic traits and decline in diversity (McGraw et al 2013). Overharvesting can negatively effect plant size. A study that looked at herbarium records of American Ginseng, found that there were significant declines in plant sizes over 150 years and determined that continuous overharvesting of older and larger plants was the cause of this decline (Cruse-Sanders and Hamrick, 2004).

The focus of this study is an American Ginseng population which had more than 300 individuals at time of census, located in Buncombe County, North Carolina. The plants in this area were wild, not planted, and had minimal disturbance except for herbivory by deer. In this study, microsatellite loci of individual plants were analyzed to determine the genetic diversity within the population and compare it to previous studies on regional populations. The author of this paper hypothesized that the focal population would have higher genetic diversity relative to the regional populations because it is so large. I expect more genetic diversity in reproductive or larger plants, as a result of heterosis or dispersal of younger stages by bird activity.

Methods

2.1 Collection and DNA extraction

Leaflet samples (N = 163) were collected from one population, hereafter called LK, in Buncombe County in summer 2022; leaflets were only collected from individuals with more than one compound leaf. Morphological data, including plant height, number of leaves, number of leaflets, and reproductive status, were also collected for each individual for later correlations between demographic and genetic variables.

Leaflets were frozen at -4ºC until DNA extraction began. DNA extraction was performed using one of two kits: a Qiagen DNeasy Plant Kit™ or an Invitrogen PureLink Plant Total DNA Purification Kit™, following their respective protocols. The quality of the DNA was then determined using a Nanodrop ND-1000 Spectrophotometer™. If DNA purity or yield was low, a standard ethanol precipitation was performed.

2.2 PCR

Polymerase chain reaction was performed to amplify 7 previously-published loci; B 011, B119, C009, C105, D227, C202, and D114 (Young et al. 2012). A PCR cocktail was made for each locus, containing a total of 13 µl, diluted with PCR water: 1X GoTaq

Master Mix™ with 4 ng each of forward and reverse primer and 5 µl of sample DNA (5 - 30 ng/ul). A T100 Thermal Cycler™ was set to specific conditions. First, the samples heated at 94 ºC for 2 min, then they completed 35 cycles of 40 s at 94 ºC, 40 s at 56 ºC, and 60 s at 72 ºC. A final extension of 10 min at 72 ºC was used, and samples were stored at 4 ºC until electrophoresis.

Table 1. Microsatellite loci information (from Young et al. 2012), including locus name, fluorophore dye, microsatellite repeat number (trinucleotide or tetranucleotide), and allele size range.

Locus	B 011	B 119	C009	C 105	C 202	D 114	D 227
Dye	6FAM	VIC	NED	PET	6FAM	VIC	NED
Repeat	tri	tri	tri	tri	tri	tetra	tetra
Size Range	113-306	160-226	176-218	195-273	199-243	180-375	150-218

2.3 Gel electrophoresis and multiplexing

Gel electrophoresis was performed on samples to verify success of PCR amplifications. Samples of each locus were run on 0.5% agarose gels, then visualized in a UVP BioDoc-It^2 Imager™. Multiplexing of samples was done in order to combine loci into groups by fluorophore (Table 1). Samples were sent to the North Carolina State University Genomic Sciences Laboratory for fragment analysis.

Figure 1. Agarose gel with PCR-amplified DNA fragments, many of which show multiple alleles. The left lane in each row is an Invitrogen™ 100 bp ladder, and many lanes show heterozygosity (more than one band).

2.4 Data analysis

Data for the comparison was collected from 11 populations of American Ginseng in the region by our research team in previous years (Figure 2.). Microsatellite fragments were analyzed in Geneious Prime v. 2022.1.1. Peaks were determined based on visual and qualitative characteristics: height of peak, the length of the DNA based on the ladder, locus information, and bins created from previous samples (Table 1.). Allele data for the focal population and 11 other populations were combined in R Studio to test the hypothesis (R studio Team, 2020). Genetic diversity was analyzed using the *polysat* package (Clark, 2011). An allelic diversity test was performed in order to determine the total number of unique alleles within the population. A second diversity test calculated genotype frequency statistics using a gene matrix to combine alleles for individuals (Clark, 2011). A Principle Component Analysis was performed on the 12 populations to test genetic relatedness. Intrapopulation diversity was examined using two demographic groups: reproductive status (flowering vs non flowering) and size class (2, 3, or 4 leaves), using the same allelic and genotypic tests above.

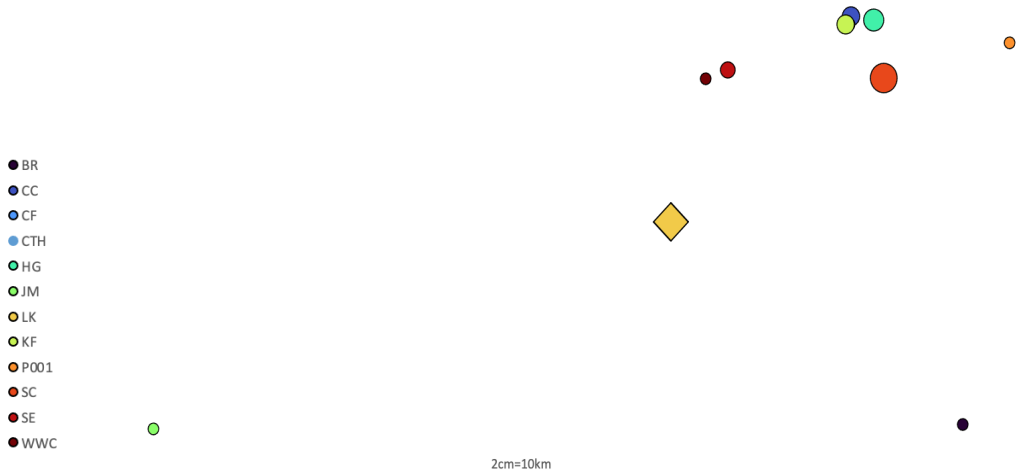

Figure 2. Location of 12 populations in relation to each other. Population size at census, if known, is indicated with symbol size; CC = 69, CF = 40, HG =146, JM = 48, KF = 58, LK = 300, P001 = 24, SC = 221.

Results

The focal population (LK) of this study was the most genetically diverse of the 12 known populations in the region according to the genotypic (Table 2) and allelic (Figure 3)

diversity tests. A Principle Component Analysis shows LK had the most intrapopulation diversity and is more distinct from 10 of the 11 populations (Figure 4). The SC population had the highest amount of allelic diversity, while LK had the second highest (Figure 3). There was overlap of SC individuals with the LK individuals, indicating genetic relatedness between the two populations (Figure 4). The 11 other populations were clustered together, meaning that there was less genetic diversity among them. Genetic diversity was higher in plants with 3 leaves than in plants with 2 leaves (Figures 5, 6). Flowering plants had more genetic diversity than non flowering plants (Figures 7, 8).

Table 2.Genotypic diversity of 12 populations in western North Carolina. LK is the focal population of this study.

Population	BR	CC	CF	CTH	HG	JM	KF	LK	P001	SC	SE	WWC
Genotypic variation	3.5	3.5	4.0	3.6	3.7	3.5	3.0	4.8	3.1	4.1	3.2	3.4

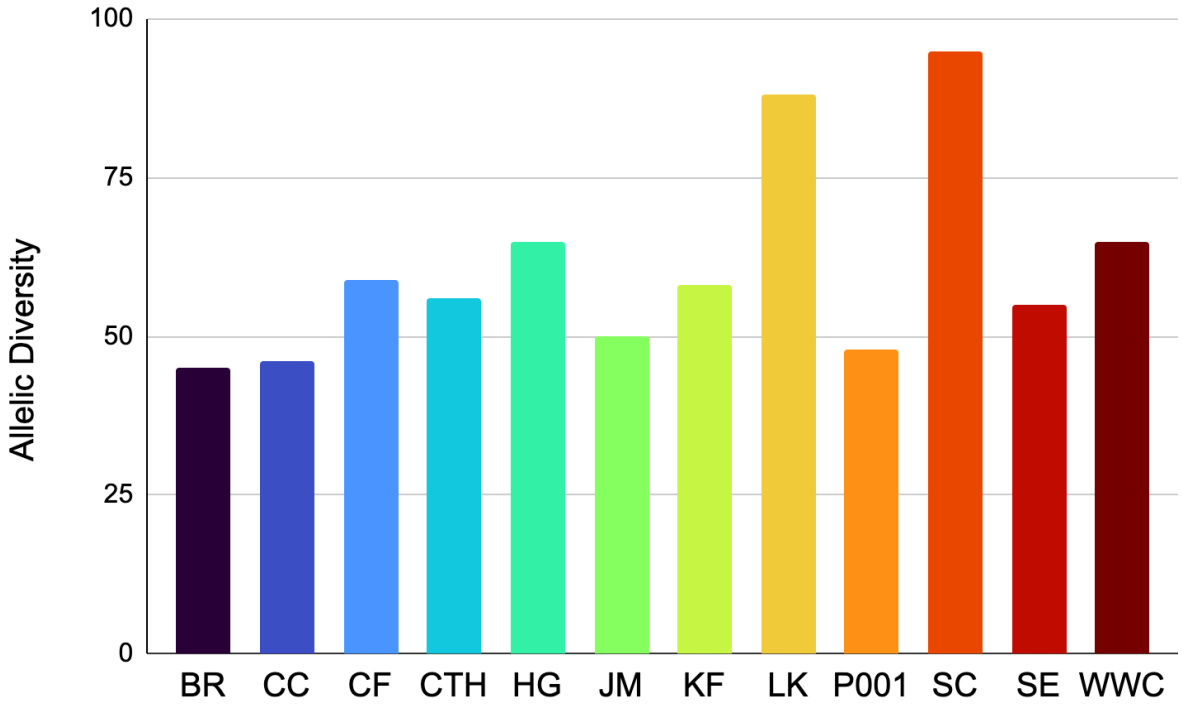

Figure 3. Allelic diversity of 12 populations in western North Carolina. Population SC had the highest level of allelic diversity and population LK had the second highest.

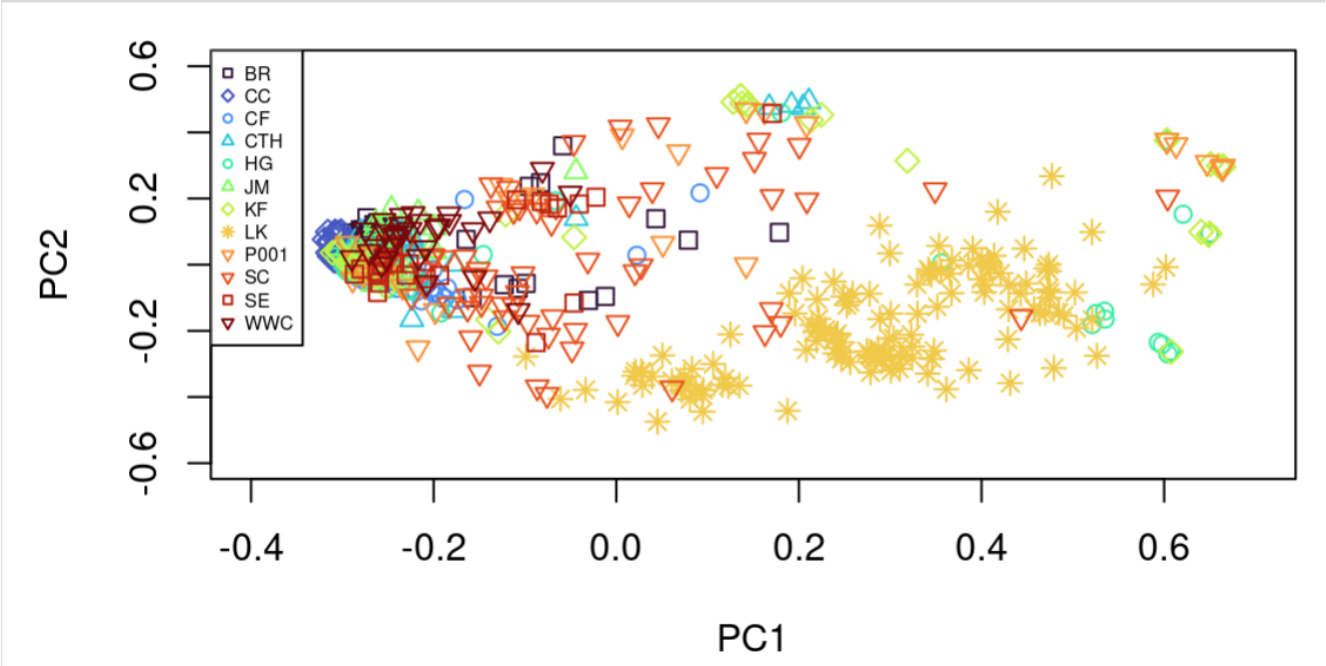

Figure 4. Principal Components Analysis plot showed genetic relationships among individuals from 12 western North Carolina populations of American Ginseng. Yellow stars represented the LK, focal population, and red upside down triangles represented the SC population.

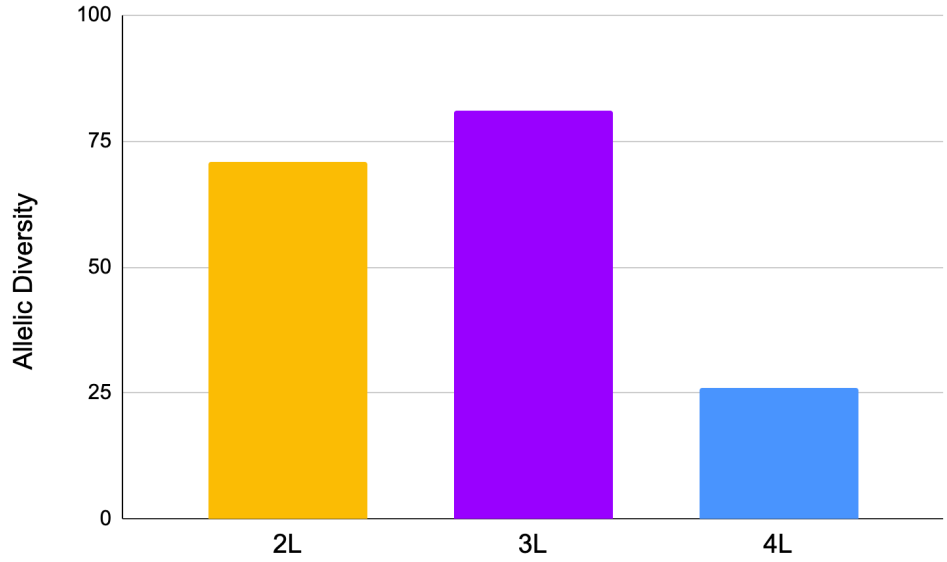

Figure 5. Allelic diversity of individuals in different size classes in population LK (2L = two leaves, 3L = three leaves, and 4L= four leaves). Plants with three leaves have the highest allelic diversity.

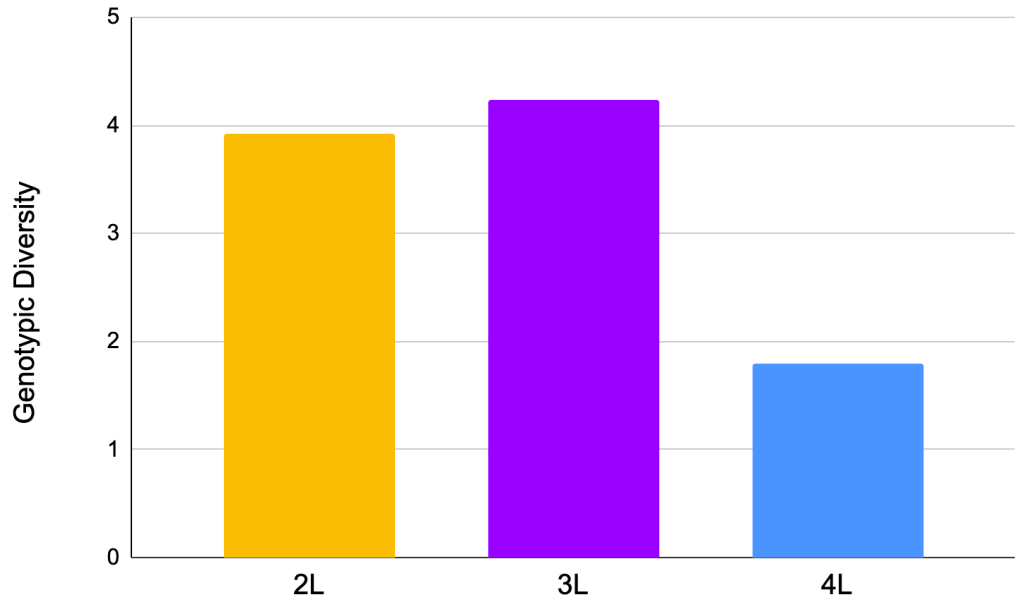

Figure 6. Genotypic diversity of individuals in different size classes in population.(2L = two leaves, 3L = three leaves, and 4L= four leaves). Plants with three leaves have the highest genotypic diversity.

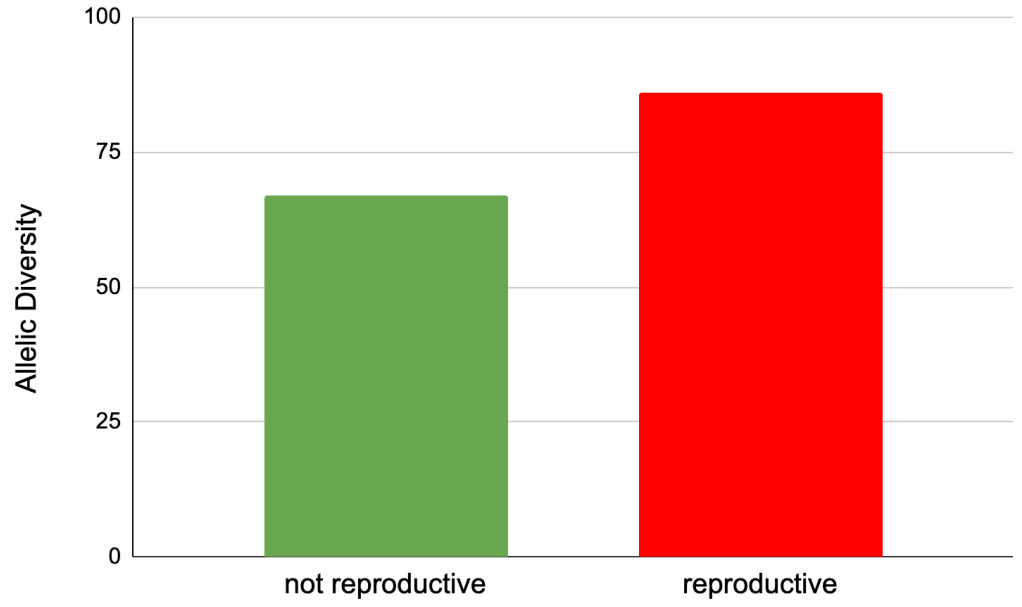

Figure 7. Allelic Diversity of reproductive and nonreproductive plants in population LK. Reproduction was characterized by plants that had flowers. Reproductive plants had the highest allelic diversity.

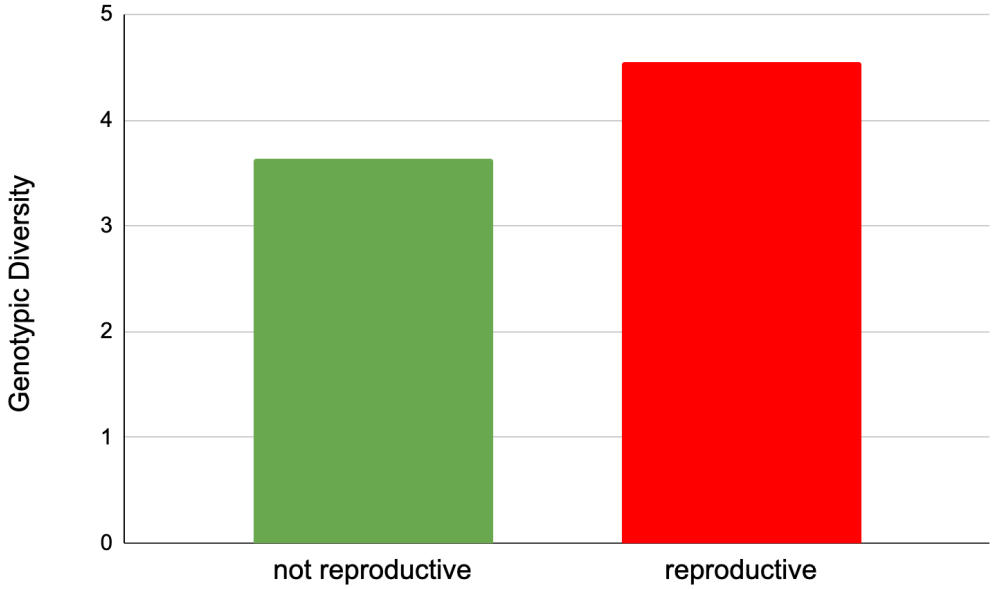

Figure 8. Genotypic diversity of reproductive and nonreproductive plants in population LK. Reproduction was characterized by plants that had flowers. Reproductive plants had the highest genotypic diversity.

Discussion

Overall, the population LK was the most genetically diverse population compared to the 11 other regional populations, supporting the initial hypothesis. This is likely because it is the largest population in the study and seems to have escaped major poaching events, as seen in (Leimu et al. 2006) and (Ilves et al. 2013). Population SC had the highest allelic diversity presumably because it is the second largest and a somewhat isolated population. These findings are similar to a pattern found in Lammi et al. who determined a positive correlation between genetic diversity and population size in a European perennial plant that experienced fragmentation (Lammi et al. 1999).

The size class with the most genetic diversity was plants with three leaves. A similar pattern was observed in a study that looked at genetic diversity of harvested and protected populations of American Ginseng in the southeastern United States. This study found that large reproductive plants had higher genetic diversity (Cruse-Sanders and Hamrick, 2004). The four-leaved plants in LK show very low genetic diversity overall which might be because the amount of four leaved plants counted and collected at census time was low. A larger sample size of plants would be needed for further comparisons. Flowering plants had more genetic diversity than non flowering plants in population LK. Genetic diversity in populations can effect reproduction; as seen in a

study on an endangered European perenial, Ilves et al. 2013, found low levels of genetic diversity as a factor contributing reproductive characteristics: seed production and germination ability (Ilves et al. 2013). The high genetic diversity in reproductive plants paired with the large population size plants might mean that LK has high levels of fitness and should be a conservation priority to sustain its diversity.

More research on this population could be beneficial to determine the most effective conservation approach. An important next step for population LK is determining effective population size. The reproductive group had more genetic diversity, but it is crucial to understand how many of those flowering plants make fruits and contribute to the next generation. This could tell us information about the future genetic diversity and persistence of this population (Reed and Frankham, 2003). This large genetically diverse LK population could be used as a source of seeds for restoring extirpated sites or augmenting existing populations. Anderson et al. 2002, stressed the importance of sourcing seeds from lots of healthy individuals from many local wild populations to help cultivate and restore the fragmented populations. They said that most of the cultivated populations of American Ginseng came from a few plants in Wisconsin, and most of them were siblings (Anderson et al. 2002). Population LK should continue to be monitored to study long term genetic and morphological information to help conserve this large population. Rare and endemic species are at higher risk of extinction than related non endangered species (Reed and Frankham, 2002). American Ginseng falls into this category and population genetics can help determine risk of endangerment and help population specific conservation approaches. It is important that future conservation research uses both genetic information and population demographic information to make informed assessments and efforts are species and population specific.

Acknowledgements

I thank Dr. R. Graham Reynolds for access to the managed forests. The University of North Carolina at Asheville Undergraduate Research Program provided support, and I appreciate assistance from labmates Michelle Paredes, William Gay, and Mars Zappia. KF samples were collected by Kat Brown, Grace Dougherty, Kiersten Keith-Pederson, and Audrey McHenry, and data from other populations was collected by Dr. David Clarke, Dr. Jonathan Horton, Dr. Jen Rhode Ward, and especially Dr. Alisa Hove (Warren Wilson College). I would like to thank committee members Professor Caroline Kennedy and Dr. Matthew Greene, and Dr. Jen Rhode Ward for helping me every step of the way as my research advisor.

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