University of North Carolina Asheville
Journal of Undergrad Research
Asheville, North Carolina
December 2018

Genetic Diversity of American Ginseng (*Panax quinquefolius*) Populations in western North Carolina

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

Faculty Advisor: Dr. Jennifer Rhode Ward

Abstract

Examining the genetic diversity of rare and threatened plant species can give insight into how environmental factors and human forces influence plant populations. Environmental and human activities have been correlated with reduction in population size and extirpation. American ginseng (*Panax quinquefolius*) is an understory herb that has been threatened by overharvesting pressures. This species has been exploited due to its medicinal uses and has been viewed as a panacea. In this study, genetic diversity was analyzed from seventeen different populations in western North Carolina, two populations from Virginia, and a commercial seed. DNA was extracted from leaflets, and PCR was performed for seven microsatellite loci. Overall populations exhibited little genetic variation, with pairwise F_{ST} values ranging from 0.0 to 0.05. However, pairwise comparisons ranged from low to moderate differentiation with few comparisons of 0.15 to 0.25 indicating great genetic differentiation. Allelic and genotypic diversity values were moderate and varied among populations. Mantel tests of geographic and genetic distance indicated that populations that are physically closer to one another and are more related due to metapopulation dynamics. Mantel tests of chemical and genetic distance indicate that there is a relationship between chemotype and genotype but only accounting for 10% of the variability explained by epigenetics and environmental factors. Low genetic diversity and population similarity is influenced by a variety of factors such as poaching, gene flow and fragmentation of historically larger populations. Allelic and genotypic diversity could be explained by overharvesting and habitat fragmentation of populations. Insight into the dynamics of genetic diversity of these populations will allow additional valuable information in the conservation and restoration efforts of for this ecological and medicinally important plant.

1. Introduction

Genetic diversity studies have been conducted on rare and threatened species to gain insight into how human activities and environmental factors influence plant survival. Environmental and anthropogenic disturbances of habitats influence the structure of plant populations and functionality[1]. Individual plant populations' survival and adaptability to environments can depend on the amount of genetic variation they contain. Plant fitness has also been directly correlated with the size of populations, where reduced population sizes exhibit less genetic diversity[2,3]. Reduced population sizes as well as fragmentation and isolation of populations are attributed to lowering gene flow, dispersal of seeds and pollen by animals, and inbreeding of populations[4]. A population's genetic diversity is easily affected by many factors that are not limited to natural disturbances. Harvesting pressures caused by human disturbances prompt changes in the homozygosity of the genetic structure and lower genetic diversity at a population level[5]. In addition to harvesting pressures, human influence is seen in global climate change, population fragmentation, and management of plant resources[4]. These influences have been shown to effect plant survival and decrease the abundancy and density of a population[4]. Additionally, geographic distances of plants are correlated with genetic diversity by interbreeding and outbreeding depression[6]. Plants within the same population would be expected to be similar because of

interbreeding and populations of different geographic regions would be expected to be different due to less interbreeding. Natural and anthropogenic influences on the genetic diversity of threatened and endangered plants are extremely relevant in the proliferation of a species and are important to understand to prevent endangerment and extinction of a species.

Genetic diversity studies on native and endangered plants have been conducted in various demographic regions. Studies conducted on harvested rare and Southern Appalachian plant species have shown a decreasing amount of genetic diversity in: *Calamagrstis cainii (*Cain's reedgrass*), Carex misera* (wretched sledge), *Geum radiatum* (Spreading avens), *and Trichophorum cespitosum* (Tufted bulrush)[4]. The low genetic diversity was found these plants and could be explained by factors of low gene flow, fragmentation, human disturbance, and the region each species originated[2]. Reduction of the genetic diversity in these plant species are associated with long-term decline of diversity[2]. Further studies on European populations of plant species *Salvia pratensis* and *Scabiosa columbaria* were shown to exhibit rapidly declining populations that were linked to genetic diversity, phenotypic variations, and population size[2]. These studies on several different rare plants differ greatly in studies conducted on American ginseng. Results of these studies have indicated that American ginseng are more likely to maintain genetic diversity despite their wide geographic range.

American ginseng (*Panax quinquefolius)* is an understory herb that has been harvested for decades and sought after for its medicinal benefits from ginsenosides. Ginseng is seen by many as a panacea, and wild-harvested roots can be sold for more than $500 per dry lb[7]. Issues concerning decline of American ginseng populations and variation of genetic diversity have been investigated as early as the 1770s[8]. Studies have shown that overharvesting, habitat fragmentation, and environmental degradation play a role in reducing population size and genetic diversity in American ginseng[7,9,10,11]. Overharvesting has become a prominent issue because of American ginseng's profitability in illegal and legal markets, resulting in harvesting pressure on American ginseng. Human activities increase pressure and isolation on plant populations and have been linked to the reduction of genetic diversity amongst populations, making them less adaptable to environmental changes[4]. In efforts to preserve and protect American ginseng from extinction, it was listed in Appendix II of the Convention of International Trade in Endangered Species of Wild Flora and Fauna in 1975 and has remained there since[13]. Various studies on genetic diversity of American ginseng have been conducted due to potential endangerment and extinction.

Studies of genetic diversity in American ginseng have been investigated in Canadian populations to provide information on restoration and preservation strategies. Genetic diversity studies of eastern Canada populations of American ginseng in previously glaciated areas exhibited a number of similarities to populations in the eastern United States. Significant variation was found among these populations, but a true genetic structure could not be obtained[13]. However, polymorphic DNA showed similarities to markers studied in some West Virginia populations[13]. Although, there was no clear genetic structure found for American ginseng populations in these Canadian populations information on genetic variation will allow for estimations of population structures in this region and give more insight into genetic patterns and trends in this demographic region. Differences in protected and unprotected American ginseng populations have also been extensively researched in this region, showing protected plants exhibited more genetic variation and allelic diversity than unprotected populations of plants reflecting a reduction in population size and, consequently, diversity's due to human and habitat disturbances[4].

In this research study, genetic diversity of *Panax quinquefolius* was analyzed for genetic diversity in western North Carolina and Virginia populations, and commercial seed. The expected outcomes for this study is high genetic diversity exhibited in western North Carolina populations.

2. Methodology

2.1 Collection and DNA Extraction

Plants from 17 populations of American ginseng were identified, tagged, and collected in western North Carolina in the summers of 2014-2018. Morphological data were collected from plant samples, and leaflets were harvested and stored in vials of silica gel in -80° freezer. Commercial seedlings of an unknown geographic origin were stratified, germinated, and harvested as seedlings; their DNA was then extracted. Plant samples were also collected from a disjunct populations in northeastern Virginia. DNA was extracted from harvested leaflets using a Qiagen DNEasy Plant MiniKit ™ with modifications including the use of warmed buffers and additional centrifugation during final steps.

Table 1. Population regions are shown with their respective county in North Carolina and Virginia. There are three different regions in Buncombe County that samples were collected. The commercial seed is represented by WI and the origin is not known.

Populations	County
CC	Buncombe 1
CF	Buncombe 1
HG	Buncombe 1
KF	Buncombe 1
PC	Buncombe 2
SC	Buncombe 2
P001	Buncombe 3
FAPH15	Carolina, VA
FAPH14	Carolina, VA
MP	Jackson
CB	Jackson
CH	Jackson
FG	Jackson
JC	Jackson
RB	Jackson
DF	Jackson
HC	Macon
MC	Macon
LS	Macon
WI	Commercial

2.2 Polymerase Chain Reaction

PCR was set up for 7 previously published loci[14]: B011, B119, C009, C105, C202, D134, and D227. Loci B011, B119, C009, C105 and C202 contained tri-nucleotide repeats, while, loci D134 and D227 contained tetra-nucleotide repeats. Each reaction was 13 µL total, including 6.5 µL 5' Master Mix©, 0.4 µL forward fluorescently labeled primer, 0.4 µL reverse unlabeled primer, 0.7 µL PCR water, and 5 µL of DNA extract. Thermocycler running conditions were set as follows: 2 min at 94°C; 35 cycles of 40 s at 94°C, 40 s at 56°C, and 60 s at 72°C;and a final extension of 10 min at 72°C.

2.3 Gel Electrophoresis and Fragment Analysis

Standard gel electrophoresis procedure was followed, with 6 µL of each PCR product mixed with 2 µL of loading dye and separated on a 1% agarose gel. Samples were run against a 100 bp ladder™, and gels were viewed on a UVP BioDoc-It^2 Imager. Successful samples were multiplexed with 5 µL of PCR product combined for each locus. Mixes were set up as follows: B011 (6FAM), B119 (VIC), C105 (PET), COO9 (NED) or C202 (6FAM), D114 (VIC), D227 (NED). Then, 2 µL of multiplexed PCR product and 3 µL of PCR H_2O were mixed with 10 µL of hi-di formamide/GeneScan 500 ladder™ solution. Then 10 µL of hi-dye and GeneScan 500 bp ladder were added to each tube of multiplex PCR product and PCR water. Multiplexed samples were sent to the DNA Analysis Facility on Science Hill (Yale University) for fragment analysis.

2.4 Data Analysis

Peak data were imported into Geneious© version 11.2. Peaks were analyzed and binned into appropriate call numbers that fit trinucleotide and tetranucleotide repeats for their locus ranges. Peak sizes were then exported into Excel as .CSV files and used to make text files for R-Studio analysis. Population genetic parameters were analyzed in the *polysat* package in R-Studio[15]. Differences in fragment lengths were analyzed within and among populations. Populations genetic parameters were calculated using *polysat* package in R-Studio version 3.5.1. Correlations with geographic or chemotypic variables were calculated using *vegan* package in R-Studio.[15]

3. Results

It is important to note that one locus had difficulty amplifying as a result of a null allele Pairwise F_{ST} values are shown (table 1) for 10 populations from western North Carolina, and Virginia, along with the commercial seeds (WI). According to Wright's F statistics, values from 0.0 to 0.05 indicate little genetic differentiation, values from 0.05 to 0.15 indicate moderate genetic differentiation, values from 0.15 to 0.25 indicate great genetic differentiation, and values above 0.25 indicate very great genetic differentiation[16]. An overall F_{ST} of 0.0143 across all populations indicating little genetic differentiation. Allelic diversity and genotypic diversity of individual populations are shown in figure 1 and figure 2, respectively. A G_{ST} value of 0.1414 was found across all 7 loci. The G_{ST} statistic values of 0 indicate little genetic variation, and values close to 1 indicate high genetic variation. Mantel statistic showing the geographic variability relationships between populations resulted in a P-value of 0.0001.

3.1 Tables and Figures

Table 1. F_{ST} values in pairwise comparison shown against populations. Comparison values are shown with little genetic difference values from 0.0 to 0.05, moderate genetic difference values from 0.05 to 0.15, and great genetic difference values from 0.15 to 0.25.

	CC	CF	FAPH15	HC	KF	MC	MP	P001	PC	RB	SC	WI
CC	0	0.1028	0.0704	0.0532	0.0398	0.0692	0.0771	0.1685	0.0551	0.1858	0.0335	0.1069
CF	0.1028	0	0.1420	0.1200	0.0598	0.1031	0.1383	0.2394	0.1289	0.1808	0.0678	0.1773
FAPH15	0.0704	0.1420	0	0.0621	0.0953	0.0726	0.1445	0.1210	0.0948	0.1729	0.1029	0.1010
HC	0.0532	0.1200	0.0621	0	0.0801	0.0693	0.0689	0.0900	0.0628	0.1267	0.0582	0.0848
KF	0.0398	0.0598	0.0953	0.0801	0	0.0575	0.1006	0.0969	0.0564	0.1131	0.0607	0.1260
MC	0.0692	0.1031	0.0726	0.0693	0.0575	0	0.1001	0.0684	0.0709	0.0833	0.0926	0.0558
MP	0.0771	0.1383	0.1445	0.0689	0.1006	0.1001	0	0.1166	0.0709	0.1204	0.0664	0.1259
P001	0.1685	0.2394	0.1210	0.0900	0.0969	0.0684	0.1166	0	0.0969	0.1610	0.1031	0.0855
PC	0.0551	0.1289	0.0948	0.0628	0.0564	0.0709	0.0709	0.0969	0	0.1443	0.0505	0.1150
RB	0.1858	0.1808	0.1729	0.1267	0.1131	0.0833	0.1204	0.161	0.1443	0	0.1459	0.1006
SC	0.0335	0.0678	0.1029	0.0582	0.0607	0.0926	0.0664	0.1031	0.0505	0.1459	0	0.1269
WI	0.1069	0.1773	0.1010	0.0848	0.1260	0.0558	0.1259	0.0855	0.1150	0.1006	0.1269	0

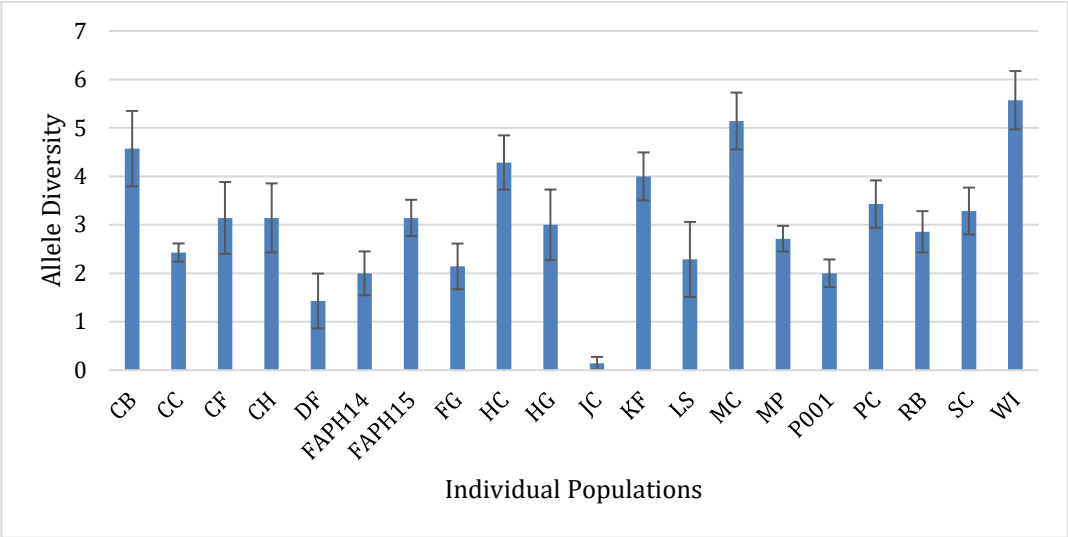

Figure 1. Allelic diversity averages with +/- 1 standard errors are shown against individual populations.

Figure 1 Shows allelic diversity amongst each individual population with allelic diversity averages and +/- 1 standard errors. Allelic Diversity varied among populations and regions.

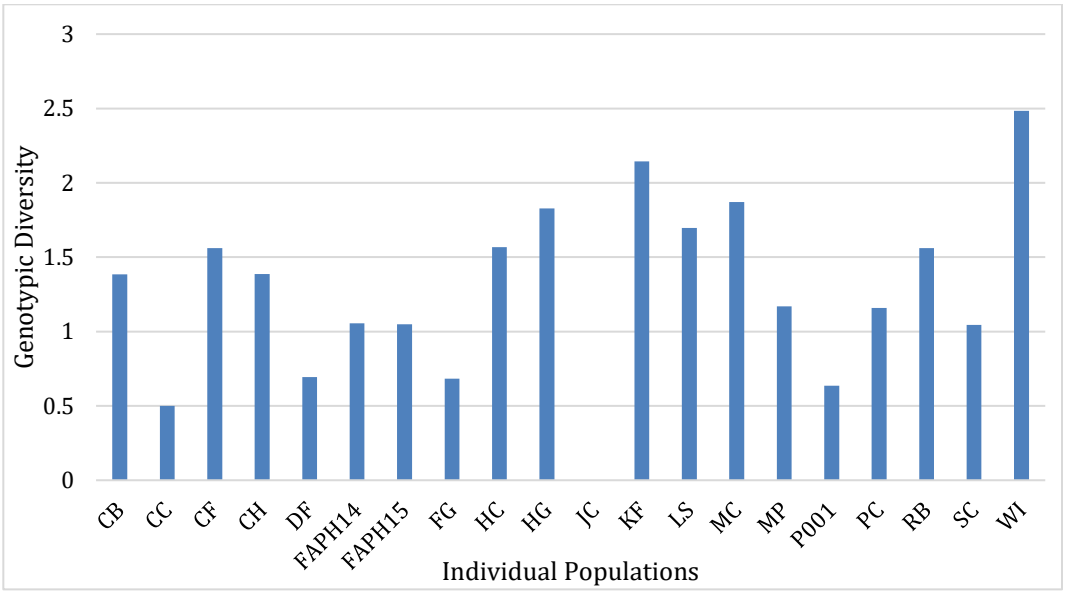

Figure 2. Genotypic diversity values shown with averages against individual populations.

Figure 2 Shows genotypic diversity amongst individual populations. Genotypic diversity varied among individual populations and regions.

4. Discussion

Populations were not as genetically different as expected. Most populations were shown to have little genetic differentiation such as KF and CC, which can be explained by both populations originating from the same region. Populations of similar origin should be less genetically different. Genetic diversity and geographic distances are

related by factors of interbreeding and outbreeding.[6] A few populations were only shown to contain great genetic difference such as P001 and CF. These populations did not originate from the same region; therefore, it would be expected that these plants would be more genetically different. Plants that are originated from the same region are more likely to interbreed and result in more homozygosity within these plant populations[6]. The little genetic differentiation could also be explained by factors such as current gene flow caused by human disturbance and seed dispersal by animals, or recent fragmentation from a previously large and contiguous population. Wisconsin commercial samples (WI) were shown to have little differentiation from VA plant samples. The Wisconsin samples as well as the VA samples (FAPH) have an unknown origin of their seeds. Results of allelic and genotypic diversity for individual populations indicate that populations with more alleles and more allele combinations are more adaptable to environmental change. Populations with lower allelic and genotypic diversity are less capable of adapting to environmental changes and decrease chances of plant fitness. A decrease in allelic and genotypic diversity could be attributed to human disturbances such as overharvesting of productive plants and habitat fragmentation. G_{ST} determines genetic variation at each loci and estimates the genetic structure within populations.[4] G_{ST} determine that populations were shown to have little genetic variation. G_{ST} and F_{ST} values are similar in determining genetic variation similar and confirmed little genetic variation within populations. Little genetic variation in G_{ST} is attributed to similar factors of $F_{ST,}$ including human disturbance causing harvesting pressures, low gene flow, and fragmentation. Mantel tests testing relationships between variables of geographic and genetic distances indicated that populations that are physically closer to one another are more related. This could be explained by metapopulation dynamics where populations that are separated but are interacting with one another. Chemical and genetic distance variables analyzed using the Mantel Test indicated populations that are chemically closer to one another are more related. This relationship explains only a small part of chemotypic variation given the r value. Contributing factors that could account for the rest of the variation are environmental factors and epigenetics.

Results indicate little genetic variation, but other studies on American ginseng across various regions to exhibit a significant amount of genetic diversity. Studies on American ginseng populations in southern Appalachian forests were shown to be highly structured with 50% variation among populations[4]. Methods in the study differed by the usage of chi-square tests, along with F_{ST}, to predict the significant expected heterozygosity of the plant populations[4]. However, microsatellites were not used, and populations in western North Carolina were not studied. Uncultivated and cultivated American ginseng populations in North American populations were also found to maintain high genetic diversity[17.] In addition, populations of American ginseng in previously glaciated regions in Canada indicated a significant amount of genetic variation but no clear genetic structure[17]. These studies on American ginseng indicate that there is strong history of maintaining its genetic diversity despite its large geographic region. Our results, however, contradict many of the studies that have conducted on American ginseng in that there is not a significant amount of genetic differentiation. The results of this study show more similarity with studies of various other species of plants than with American ginseng populations from other regions. A study conducted on four rare southern Appalachian plant species (*Calamagrstis cainii, Carex misera, Geum radiatum, and Trichophorum cespitosum*) found a reduction of genetic diversity in three of the species[2]. This study would support the explanation of low gene flow resulting in lowered genetic diversity found in our results. Genetic structure results indicated low variation within populations in this study, but other studies conducted on harvested and protected populations from regions of Canada, and the United States exhibited total genetic variation occurring in half of the populations[4]. Low genetic variation in these populations could be influenced by human influences such harvesting pressures and habitat fragmentation.

Some of these barriers included suboptimal amount of product in a sample or locus to send for fragment analysis. One locus studied had difficulty being amplified which could have been a result of a null allele. Null alleles occur when flanking region in nucleotide sequences vary and primers are unable to anneal during amplification by PCR[18]. In this study, further research could include obtaining samples from different populations in Western North Carolina that might provide more insight into genetic diversity of American ginseng in this particular region and comparing natural populations to additional commercial populations to look for evidence of genetic contamination. This study could also be expanded by focusing on genetic diversity of protected and unprotected populations. This would provide information on whether protection of threatened plants species, such as American Ginseng, are increasing individual plant fitness by decreasing harvesting pressures. Insight into American ginseng population dynamics can provide researchers with more information in conservation and restoration efforts of this species.

5. Acknowledgments

Thanks to Dr. H David Clarke and, Dr. Jonathan L. Horton for help with field collections. Andy Warren, Collin Perry, Kira Lindelof, Magnolia Wilson, and Sabrina Irizarry provided laboratory assistance, and Dr. Matthew Estep provided

technical advice. This work was supported by a grant from the North Carolina Biotechnological Center to Jonathan L. Horton, Jennifer Rhode Ward, and H.David Clarke.

6. References

1. Chandler J., McGraw J. 2016. Demographic stimulation of the obligate understorey herb, *Panax quinquefolius L.*,in response to natural forest canopy disturbances. Journal of Ecology. 105(3): 736-749

2. Godt M., Johnson B., Hamrick J. 1996. Genetic Diversity and Population Size in Four Rare Southern Appalachian Plant Species. Conservation Biology 10(3):796-805.

3. Godt, M., Hamrick J. 2001. Genetic diversity in rare southeastern plants. Natural Areas Journal 21: 61-70.

4. Cruse-Sanders J., Hamrick J. 2004. Genetic Diversity in Harvested and Protected Populations of Wild American Ginseng, *Panax quinquefolius* L. (Arialiaceae). American Journal of Botany 91(4):540-548.

5. Ellstrand N., Elam D. 1993. Population genetic consequences of small population size: implications for plant conservation. Annual Review of Ecology and Systematics. 24: 217-242.

6. Mooney E., McGraw J. 2007. Effects of Self-Pollination and Outcrossing with Cultivated Plants in Small Natural Populations of American Ginseng, *Panax quenquefolius* (Araliaceae). American Journal of Botany 94(10):1677-1687.

7. Robbins C. 1998. American Ginseng: the root of North America's medicinal herb trade. TRAFFIC North American Report. Number B347, Washington. D.C., USA.

8. Kalm P. 1987. Peter Kalm's travels in North America: The English version of 1770. *Translated by*A.B. Benson. Dover Books.

9. Laurance F. 1999. Reflections on the tropical deforestation crisis. Biological Conservation 91: 109-117.

10. Soule E. 1991. Conservation tactics for a constant crisis. *Science*253: 744-750.

11. Vance C. 2002. Ecological Considerations in sustainable use of wild plants. In: Jones, E.; McLain, R.; Weigand, J., eds. Nontimber Forest Products in the United States, 151-162. University Press of Kansas.

12. Anderson R., Anderson M., Houseman G. 2002. Wild American Ginseng. Native Plants Journal (Indiana University Press) 3(2):93-105.

13. Joly S., Archambault A., Pellerin S., Nault A. 2017. Genetic structure of the American ginseng (Panax quinquefolius L.) in Eastern Canada using reduced-representation high-throughput sequencing. Botany95(4): 429

14. Young J., Eackles M., Springmann M., King T. 2012. Development of tri- and tetra- nucleotide polysomic microsatellite markers for characterization of American ginseng (Panax quinquefolius L.) genetic diversity and population structuring.

15. Clark, Lindsay V and Jasieniuk, M. "POLYSAT: an R package for polyploid microsatellite analysis." *Molecular Ecology Resources* 11.3 (2011): 562-566. Web.

16. Weir B., Cockerham C. 1984. Estimating *F*-Statistics for the Analysis of Population Structure. Evolution. 38 (6): 1358-1370.

17. Schluter C., Zamir P. 2002. Genetic Diversity among Natural and Cultivated Field Populations and Seed Lots of American Ginseng (Panax quinquefolius L.,) in Canada. International Journal of Plant Sciences 163(3):427-439.

18. Chapuis M., Estoup A., 2006. Microsatellite Null Alleles and Estimation of Population Differentiation. Molecular Biology and Evolution. 24 (3):621-631.