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Correlating Allelic Diversity and Ginsenoside Variation in Wild Populations of American Ginseng (*Panax quinquefolius* L.) from Western North Carolina

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Abstract

American ginseng (*Panax quinquefolius*) is a threatened herb with medicinal effects caused by organic compounds called ginsenosides. Ginsenosides are the reason for the lucrative pharmaceutical and food market for *P. quinquefolius*. This demand has resulted in overharvesting of the plant, reducing its genetic diversity. It is proposed in this study that ginsenoside levels are directly related to a plant's genetic makeup. Genetic markers for *P. quinquefolius* were analyzed from 53 plants in five populations using seven newly developed species-specific microsatellite primers. DNA was extracted from each tissue sample, and primers were used in Polymerase Chain Reactions (PCR) to amplify microsatellite regions of the genome. PCR products were then subjected to agarose gel electrophoresis to visualize and quantify band sizes. These data were compared to existing ginsenoside data for these samples, and ANOVA was used to determine relationships between allelic diversity and ginsenoside content. It was determined that two of the five populations had higher ginsenoside concentrations than the others, and that two populations were less genetically diverse than the others. Additionally, only one primer amplified a DNA locus significantly correlated with ginsenosides. This study is important because an understanding of the genetic basis of ginsenosides can be used to reduce the amount of wild *P. quinquefolius* needed to satisfy the demand by pharmaceutical and food companies. It also can render pharmaceutical and food companies better able to label ginsenoside ingredients and ensure quality control in their products.

1. Introduction

American ginseng (*Panax quinquefolius* L., Araliaceae) is an herbaceous congener of Asian ginseng (*Panax ginseng* C.A. Meyer), which has long been valued in Asia for its medicinal properties[1]. Both *P. quinquefolius* and *P. ginseng* are used in herbal remedies and supplements to combat ailments of the cardiovascular[2], immune[3], endocrine[4], and nervous systems[5]. The biologically active properties in both species are due to organic compounds called ginsenosides[6]. Ginsenosides are saponin glycosides that exist in two types, 20(s)-protopanaxadiol and 20(s)-protopanaxatriol[1]. These differ mainly in the number of sugar groups attatched[7]. Ginsenosides common in American and Asian ginseng are Rb1, Rc, Rd, Re and Rg1, and individual plants are generally categorized based on their ratios of Rg1 to Re[8]. This categorization gives a plant its chemotype, and three main chemotypes are recognized: Re/Rg1 < 1.0, Re/Rg1 between 1 and 2, and Re/Rg1 > 2.0[1].

Ginsenosides vary in their medicinal effects[9]. For instance, Rg1, whose concentrations are higher in *P. ginseng* than *P. quinquefolius*, encourages angiogenesis which aids in wound healing[9]. Experiments have shown that geographically distinct populations of *P. quinquefolius* vary in their respective chemotypes[8,10], which is pharmacologically significant for companies trying to manufacture ginseng products with specific outcomes. However, it is unknown whether these differences are due to environmental factors, genetic factors, or both. A few

studies suggest a genetic basis for variation in medicinal compounds, such as for Turkish basil, *Ocimum basilicum* L[11] and psyllium, *Plantago ovata* Forsk[12].

The demand for ginseng as an herbal supplement has led to excessive harvesting of wild *P. quinquefolius*, which has severely reduced population size and stability[13]. Ginseng harvesting is inherently destructive because its Asian market value is based on the physical appearance of roots. Therefore, whole roots are removed from the soil, which kills the plant[13]. Overharvesting has caused this species to be listed on Appendix II of the Convention on the International Trade of Endangered Species (CITES)[6]. *Panax quinquefolius*'s listing on CITES raises the question of how to conserve populations without destroying this economically important market. Elucidating relationships between genotypes and chemotypes could allow ginseng cultivars, or domesticated plants of a known genotype[14], to be developed, reducing overharvesting from wild populations[15]. Schlag and McIntosh attempted to determine such relationships using random amplified polymorphic DNA (RAPD) loci[1]. However, intergenic DNA regions called microsatellites are more accurate genetic markers to be used in this endeavor[16] because they are species-specific and amplify only introns[17], which are not under natural selection pressure[18]. Therefore, this research is a continued investigation of the results of Schlag and McIntosh using more reliable methodology and a different set of populations known to exhibit relatively high genetic diversity[19].

In this study, microsatellites were amplified from five Western North Carolina populations for plants whose ginsenoside composition had been previously determined[20]. It was hypothesized that variations in total root ginsenosides have a genetic basis, and that Re, found in only a few plants, is associated with a single microsatellite locus. This study is unique and important in its use of microsatellites to determine the genetic basis for *P. quinquefolius* ginsenosides. The results could be used to create cultivars of ginseng with desired chemotypes[1] and can be used by companies to more accurately label the ginseng compounds in their products[20].

2. Materials and Methods

2.1 Chemotype Determination

Fifty-three leaf and/or stem tissue samples of *P. quinquefolius* were collected from five western North Carolina populations in 2009-2011, then stored in a -80°C freezer). Ginsenosides were extracted from dried roots and leaves using a 50:50 ethanol:water solution, then dried at room temperature[20]. Chemotypes were determined by High Performance Liquid Chromatography (HPLC) (PerkinElmer Series 200, Shelton, CT) using a 3 µm, 150 x 3.0 nm, C18 column (Thermo Scientific, West Palm Beach, FL)[20]. Samples of the ginsenosides Rb1, Rb2, Rc, Rd, Re and Rg1 (purchased from Indofine Chemical Co., Inc., Hillsborough, NJ) were run as standards against the extracted samples[20]. For the 2009 and 2010 leaf samples, HPLC was performed at the Asheville-Buncombe Technical Community College Natural Products Laboratory while for the 2011 samples, HPLC was performed by the UNC Asheville Biology Department. Ginsenoside concentrations are shown in Table 1.

Table 1. Leaf and/or stem Re and total ginsenoside concentrations as determined by previous students, as well as ages and number of *P. quinquefolius* plants from populations in western North Carolina.

Site	Number of samples	Plant ID	Age (years)	Re (µg/g)	Total (µg/g)
CPH	3	390	15	0.0000	29.7222
CPH	.	393	18	0.0000	49.4237
CPH	.	412	25	2.8328	43.2116
LB	16	12	16	0.0000	45.9090
LB	.	16	13	0.0000	20.2380
LB	.	27	19	0.0000	28.7730
LB	.	31	21	0.0000	27.0070
LB	.	49	12	0.0000	20.6740
LB	.	64	15	0.0000	23.9750
LB	.	73	7	0.0000	13.9760
LB	.	83	12	0.0000	16.6090
LB	.	86	16	0.0000	35.7760

LB	.	88	9	0.0000	11.9810
LB	.	94	13	0.0000	30.0990
LB	.	205	12	0.0000	30.7000
LB	.	227	22	0.0000	8.8414
LB	.	K	14	0.0000	14.0207
LB	.	N	12	0.0000	49.7090
LB	.	Y	8	0.0000	24.6260
MC	5	521	6	8.8629	39.2491
MC	.	522	12	13.8904	86.3212
MC	.	525	9	30.5704	62.5209
MC	.	526	20	38.9773	129.3469
MC	.	528	13	31.1122	101.2831
PC	14	13	30	0.0000	38.1620
PC	.	21	12	0.0000	29.3790
PC	.	37	11	0.0000	17.7911
PC	.	38	11	6.3040	36.2880
PC	.	44	22	0.0000	18.2370
PC	.	50	20	0.0000	23.8350
PC	.	51	11	0.0000	19.3840
PC	.	60	8	0.0000	27.6440
PC	.	257	10	0.0000	19.1863
PC	.	E	9	0.0000	22.0290
PC	.	K	9	0.0000	29.1521
PC	.	L	13	0.0000	16.8040
PC	.	R	5	0.0000	37.8614
PC	.	T	13	1.5257	12.2118
PC	.	W	21	6.0530	31.1200
SC	14	16	13	0.0000	34.3190
SC	.	18	19	0.0000	31.4010
SC	.	21	45	0.0000	29.5000
SC	.	32	13	0.0000	13.2430
SC	.	35	18	0.0000	23.6930
SC	.	40	14	0.0000	25.7670
SC	.	43	13	0.0000	21.8740
SC	.	48	7	0.0000	12.2830
SC	.	58	35	0.0000	39.5520
SC	.	62	12	0.0000	19.7490
SC	.	212	26	0.0000	73.7987
SC	.	217	13	0.0000	18.6310
SC	.	261	24	0.0000	18.4210
SC	.	BJ	.	0.0000	32.7774

2.2 Extraction and PCR of DNA

Whole genomic DNA was extracted from tissue samples listed above with Qiagen DNeasy Plant Mini kits (Qiagen, Valencia, CA), and DNA concentrations of the samples were quantified using a spectrophotometer (NanoDrop, Wilmington, DE). Ideal concentrations were around 10 ng/μL, and low or high concentrations were corrected by ethanol precipitation or adding AE Buffer, respectively. During ethanol precipitation, 1/10 the total volume of sodium acetate was first added, then 2X the total volume of at least 95% ethanol was added. Samples were stored in a -80°C freezer overnight, centrifuged at 13,000 rpm for 30 minutes, then spun in an Eppendorf Concentrator 5301(Eppendorf North America, Hauppauge, NY) at room temperature until supernatant was completely evaporated. Samples were then re-hydrated by adding 20 μL of ddH_2O.

A total of 12 newly developed microsatellite primers specifically for *P. quinquefolius*[21] were ordered from Eurofins MWG Operon (Huntsville, AL), and they were tested for their ability to amplify using Polymerase Chain

Reaction (PCR) with a small subset of DNA samples. The seven most consistently amplifying primer sets (B011, B119, C009, C105, C202, D114, and D227) were used with remaining samples. The PCR recipe was modified from King *et al.*[22] and consisted of seven µL of DNA sample, one µL each of the forward and reverse primer, and nine µL of MasterMix (5 PRIME, Gaithersburg, MD). PCR was performed using a thermocycler (BIO-RAD, Hercules, CA) with the protocol described in King *et al.*[22]

2.3 Electrophoresis of PCR Products

Gel electrophoresis was performed by running 34 PCR products at a time along with a 25 bp DNA Step Ladder (Promega, Madison, WI) in a large 2% agarose gel at 110 Volts for approximately two hours (Figure 1). Gels were photographed using the KODAK Gel Logic 100 Imaging System at an F-stop of 5 and the UV Transillumination setting. Images were edited and band sizes were quantified using Carestream MI software (Rochester, NY). Band sizes were recorded, and data were compiled in a Microsoft Excel spreadsheet.

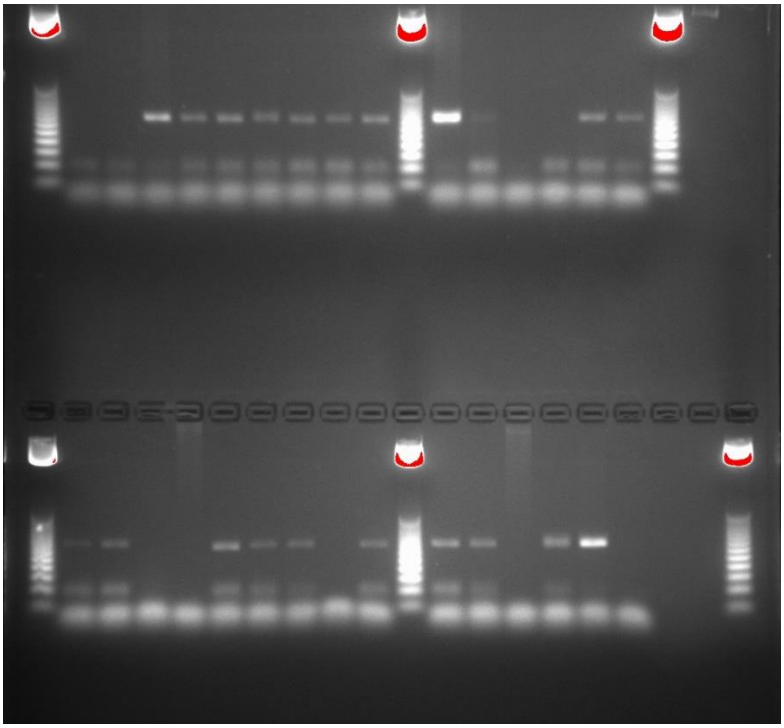

Figure 1. PCR products using primer D227 on an agarose gel.

Figure 1. PCR products using primer D227 run on a 2% agarose gel. Top row, from left to right: Promega 25 bp step ladder, LB86, LB84, LB94, PC37, PC51, PC38, PC60, PC50, CPH393, Promega 25 bp step ladder, CPH412, LBN, LB12, LB16, LB27, LB13, Promega 25 bp step ladder. Bottom row, from left to right: Promega 25 bp step ladder, LB49, PCW, PC39, PCK, PC44, PC13, SC40, SC35, SC16, Promega 25 bp step ladder, SC21, SC251, PCT, PC21, LB73, LB83, blank lane, blank lane, Promega 25 bp step ladder.

2.4 Statistical Analysis

The GenAlEx 6.5 plug-in[23] for Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA) was used to produce descriptive statistics for the genetic patterns present across the five populations (N = 53). For each population, the number of different alleles, private alleles, observed heterozygosity and Nei's genetic identity were determined. SAS software (SAS Institute Inc., Cary, NC) was used to perform a Gauss-Newton non-linear regression test (SAS Proc NLIN) to examine relationships between plant age and total root ginsenoside concentration. Analysis of variance

(ANOVA) test (SAS Proc GLM) with Tukey-Kramer *post hoc* tests were used to determine differences in total ginsenoside concentrations among populations, with population being the independent variable and ginsenosides being the dependent variable. ANOVAs were performed to relate total ginsenosides to both age and alleles at each locus. A non-parametric ANOVA was run to elucidate the relationship between population and Re levels, and a series of Kruskal-Wallis tests (SAS Proc NPAR1WAY) were used to determine relationships between alleles at each locus and total Re levels.

3. Results

3.1 Allelic Patterns Across Populations

Populations LB, PC and SC had the highest number of different alleles, private alleles and the highest expected heterozygosity (Fig. 2). Populations CPH and MC had the lowest measures for these values (Fig. 2). A pairwise population matrix of Nei's genetic identity showed that LB and PC were most closely related genetically (Table 2). Populations MC and SC were the second most closely related, and SC and PC were the third most closely related (Table 2).

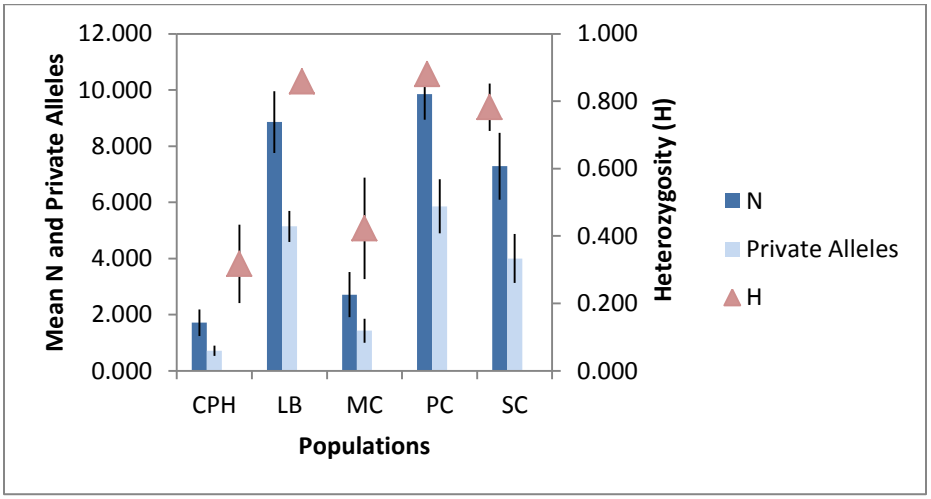

Figure 2. Allelic patterns across the five populations.

Figure 2. A plot of several allelic patterns across the five populations. N = the number of different alleles within each population, private alleles = the number of alleles unique to a single population, and H = observed heterozygosity. Means are presented + / - 1 SD.

Table 2. Pairwise population matrix of Nei's genetic identity, where a value of zero indicates that there are no common alleles between populations and a value of one indicates complete identity.

	CPH	LB	MC	PC	SC
CPH	1.000				
LB	0.139	1.000			
MC	0.000	0.044	1.000		
PC	0.115	0.257	0.144	1.000	
SC	0.113	0.060	0.172	0.167	1.000

3.2 Population Versus Ginsenoside Content

There was a significant (df = 1/68, P = 0.0001) relationship between population and ginsenoside concentrations. This analysis was done with a larger sample set than was used for allelic analyses, therefore the degrees of freedom here is higher than what would be expected from the final 53 samples. The Tukey-Kramer *post hoc* test indicated that MC exhibited the highest total ginsenoside concentrations and that CPH exhibited the second highest concentrations (Fig. 3). Populations LB, PC, and SC did not differ significantly from each other in this aspect (Fig. 3).

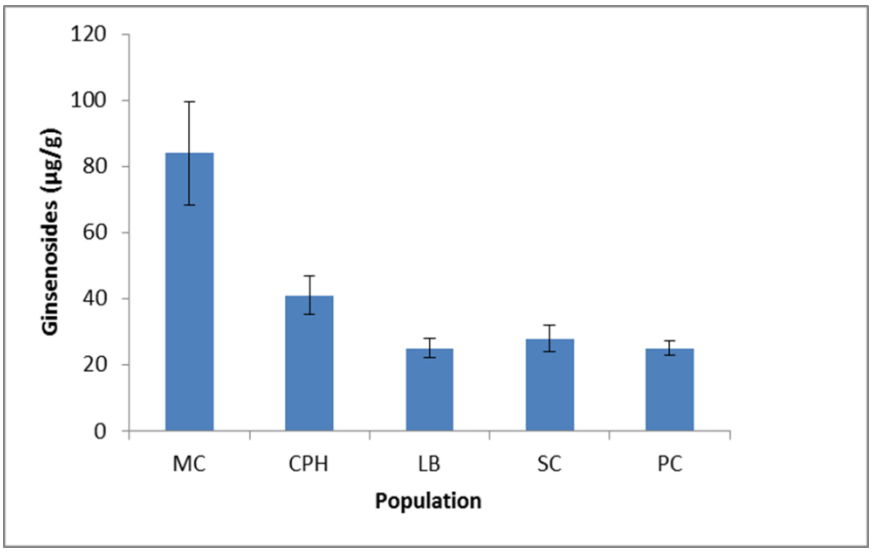

Figure 3. Populations ranked based on total ginsenoside concentration.

Figure 3. Populations ranked on their mean total ginsenoside concentration. Values are presented + / - 1 SE.

3.3 Locus Versus Age and Ginsenosides

The Gauss-Newton non-linear regression model showed a significant positive relationship between age of a plant and its total root ginsenoside concentrations (df = 1/66, P = 0.0001). From the series of ANOVAs run for each locus, it was determined that only the B011 alleles were significantly related to age ($P < 0.0003$) and to total root ginsenosides ($P < 0.0001$) (Fig. 4), while alleles from the other six loci were not significantly related to these ($P > 0.05$). The coefficient of determination was low; therefore, the variation among data points was not well explained by the model and would be better explained by untested factors.

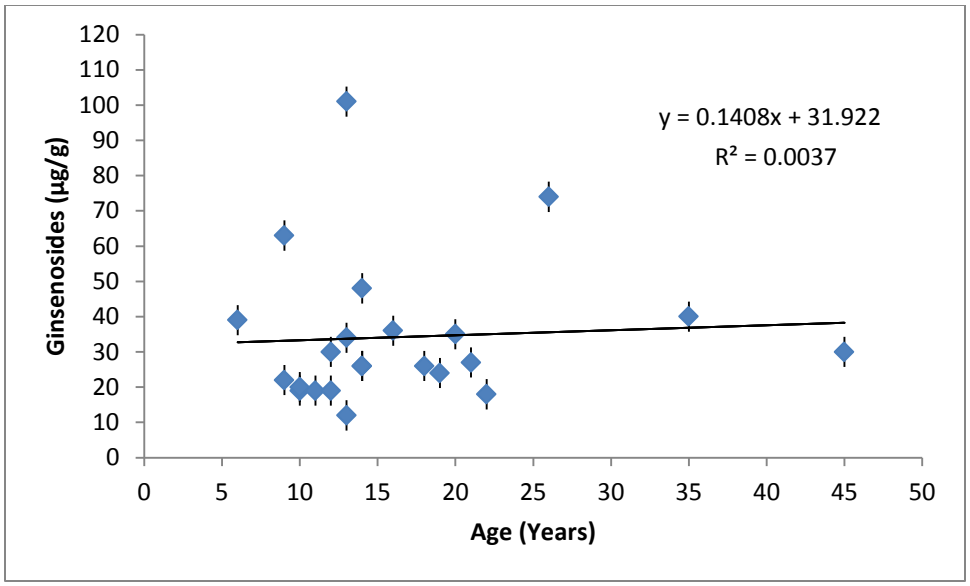

Figure 4. Age versus ginsenoside levels for alleles at the B011 locus.

Figure 4. A plot showing alleles at the B011 locus as a function of plant age and total ginsenoside levels, where each data point represents the combination of alleles for an individual plant. Values are presented + / - 1 SE.

3.4 Population and Locus Versus Total Re Concentrations

The ANOVA relating population to Re concentrations showed that the two were significantly related ($P = 0.0001$). The Kruskal-Wallis non-parametric tests showed that none of the alleles at the seven loci were significantly related to Re, however ($P > 0.05$ for all).

4. Discussion

The results of the non-linear regression model indicated that higher plant age correlated to higher concentrations of ginsenosides, which agrees with the results of other studies[24, 25]. The ANOVA and Tukey-Kramer tests comparing population and ginsenoside levels showed that populations MC and CPH had significantly higher levels of ginsenosides than the other three, which could be due to a unique genotype in those populations. Considering that they exhibited a lower number of private alleles, though, it is more likely that these populations are experiencing environmental factors that are influencing ginsenoside production. For Re, its presence or amount in plants had no genetic basis. Since populations were significantly related to Re but microsatellite loci were not, it could be possible that environmental factors[26] or other loci could be responsible for Re production.

The allele frequency statistics for the five populations showed that CPH and MC had low genetic diversity. This would suggest that these populations have historically undergone higher rates of overharvesting. However, this result could also an artifact of CPH and MC contributing fewer tissue samples used than the other three. The number of samples used from each site was solely a matter of which ones had ginsenoside data previously determined, and it in no way represented the size of the population. Therefore, future studies that are able to perform new chemotype analyses and therefore use equal sample sizes from populations could show different results. The genetic similarity of populations LB and PC as determined by the Nei's genetic identity test could mean that in the past there was at least some level of gene flow between them, and that these two populations have the potential to produce plants with similar chemotypes.

The result that B011 was the only locus to be significantly related to total root ginsenosides indicates that the allele patterns present at this locus could be diagnostic for ginsenoside production. Meaning, the allele patterns could be used to make predictions about which or how much ginsenosides a plant might produce. This has implications for

industry uses of *P. quinquefolius* as well as for future scientists. For pharmacology or food companies trying to develop plant cultivars for use in their products, this means that only one PCR primer is needed to identify plants that could produce the desired chemotype (although further examination is needed to determine the relationship between B011 and each separate ginsenoside). The same holds true for future researchers; using just primer B011 could give reliable information about chemotypes present in *P. quinquefolius* populations.

Schlag and McIntosh used RAPD markers to provide evidence for the genetic basis of chemotype of plants in Maryland[1]. Although their research created the basis for this study, there are several problems associated with using RAPDs as tools for genetic analyses. RAPD loci are unable to distinguish heterozygosity in individuals, and due to the fact that they are random polymorphic segments throughout the genome, they are not necessarily specific for introns[17]. Microsatellites, on the other hand, are specific for known intron sequences in a given species[17]. Introns are not under selection pressure and therefore can accumulate various mutations with no effect on the organism, so studying similarities among introns are useful for showing relatedness among individuals[18].

Other studies have also used molecular techniques to identify the genetic basis of other medicinal plant's effects, such as for Turkish basil, *Ocimum basilicum* L[11]. In a study very similar in concept to this one, RAPD loci for 14 genotypes of *O. basilicum* were amplified while their essential oils were quantified by gas chromatography[11]. From this, it was determined that the genotypes could be grouped into 7 distinct chemotypes[11]. A method other than polymorphic gene markers was used with phyllium, *Plantago ovata* Forsk[12]. The isozyme patterns of *P. ovata* were analyzed using polyacrylamide gel electrophoresis and it was determined that all individuals did not express the isozymes equally, and that 9 genetic loci were identified as correlating to the different expression patterns[12]. Although protein expression is indirectly related to genes, this study indicates that *P. ovata* may have a selection of genetically-based chemotypes, similarly to *P. quinquefolius*[12]. So, this study fits into the existing collection of research on the relationship between a plant's genetic makeup and medicinal compounds, and contributes to the scientific community's understanding of this relationship in *P. quinquefolius*.

The results of this study are important for *P. quinquefolius* conservation endeavors. This is because information regarding the genetic basis of ginsenosides can be used by pharmaceutical companies trying to create a product containing specific ginsenosides. If a company knows which ginsenosides they desire for their product, they are able to breed cultivars to harvest from[15]. The increase in domestic cultivars will significantly reduce the amount of wild *P. quinquefolius* needed to serve the Western market. These results are also important in that companies will be able to accurately label the ginseng chemotype used in their product, ensuring higher quality control and consumer awareness[20]. Future studies should expand the number of populations sampled in the southern Appalachians and include analyses of tissues from new individuals. It is also recommended to study environmental parameters that could affect Re production.

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