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Ginsenoside Profiles and Diversity in Western North Carolina Populations of American Ginseng (*Panax quinquefolius* L.)

Andrew J. Warren
Biology
The University of North Carolina at Asheville
One University Heights Asheville
North Carolina 28804 USA

Faculty Advisor: Dr. H. David Clarke

Abstract

American ginseng (*Panax quinquefolius* L.), an economically important plant, has been collected and cultivated for use as herbal medicine since the 18th century. A class of triterpenoid saponins, ginsenosides, comprise the most abundant and potent medicinal compounds found in ginseng. This study examined the diversity of this group of secondary metabolites in the context of the glacial refugia of the Southern Appalachian Mountains for the first time. This study was intended to clarify the relationship of the growing environment and genetics in expression of ginsenosides. We hypothesized that 1) chemotypic diversity would be distinct from previously reported values, 2) that plants with a Rg1 dominant profile would be the most commonly observed, and 3) the chemical diversity would be associated with the genetic structure of western North Carolina populations. Seventeen wild ginseng populations in western North Carolina and two from Virginia's coastal plain were sampled, then six ginsenosides (Rb1, Rb2, Rg1, Re, Rd, and Rc) in the root tissue were extracted and quantified by high-performance liquid chromatography (HPLC), with detection by UV spectroscopy. DNA was extracted from leaf samples and fragment lengths at 7 microsatellite loci were used to analyze genetic diversity. Analysis of these data found Rg1 dominant chemotypes to be the most common in the area studied and identified the ratio of Rg1/Rb1 to be of interest for further study. Further examinations of the etiology of chemical diversity of *P. quinquefolius* are necessary to breed cultivars labeled for their regional origin, phytochemical profiles and specific medicinal properties, an effort which will ideally reduce the demand for wild harvested ginseng. These analyses lay the groundwork for a more refined characterization of ginsenoside profiles found in wild populations, and support efforts to identify, cultivate, and conserve regional diversity.

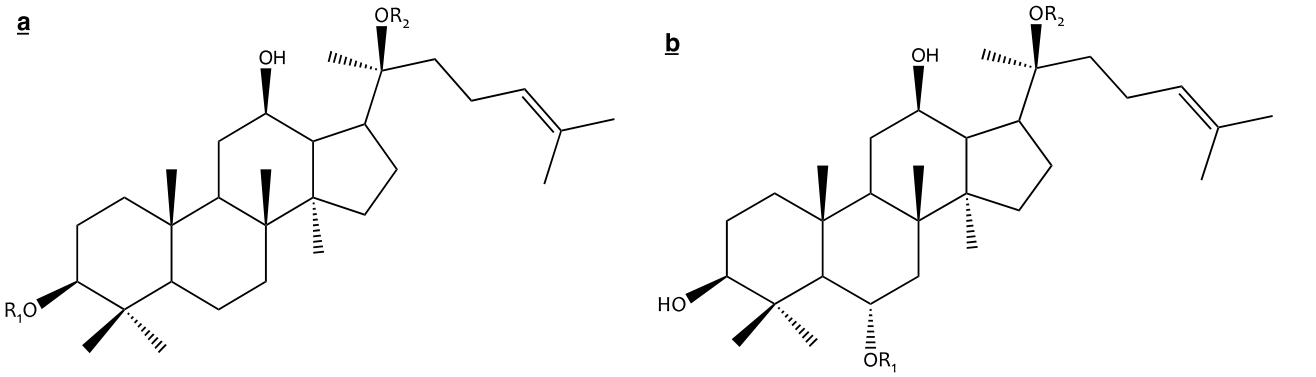
1. Introduction

American ginseng (*Panax quinquefolius* L.) is a threatened perennial understory herb endemic to eastern North American deciduous forests. Due to its value as an herbal and folk medicine ginseng has been overharvested for over two centuries. Demand for American ginseng in East Asian markets rose in response to the increasing rarity of an overharvested indigenous Asian congener (Korean ginseng; *Panax ginseng* C.A. Mey.), to the detriment of the health of wild populations[1,6,7,12,22,33]. This history is concurrent with cultivation of ginseng across its native range, which has resulted in a large flow of genetic stock between regions, and the possibility of introgression of non-native genes into apparently wild populations[41].

The medicinal value of *Panax* species is primarily derived from ginsenosides, a class of triterpenoid saponins[3,16,18]. The ecological roles of ginsenosides in *Panax* species include exerting regulatory influences on soil and foliar microbial communities and they have been shown to have diverse regulatory effects on soil and foliar microbiota, and possible anti-herbviory activity[14,16,23,35]. The numerous bioactivities of these chemicals have also endowed ginseng with a reputation as a cure-all, or adaptogen, and study of these chemicals and their actions *in vivo* has revealed a diversity of physiological effects as well as chemical profiles within *P. quinquefolius*[3,4,16,25,31,39]. Over 100 ginsenoside

species have been described[34], and phytochemical profiles can be used to identify variation within and among ginseng populations[2,17,30,32,39]. This study focused on six ginsenosides that have been shown to be abundant in *P. quinquefolius*: Rg1, Re, Rb1, Rc, Rd, and Rb2 (Fig. 1).

The ratio of Re/Rg1 has historically been used to differentiate American and Korean ginseng, establishing the ratio as the standard for chemotyping *Panax* species[26]. Although *P. quinquefolius* is typically characterized as containing large quantities of the ginsenoside Re and relatively little Rg1 prior studies have shown that such a trend does not hold true across all populations[2,17,28,29,30]. Additionally, it has been shown that the phytochemical profiles observed are strongly influenced by population origin, in particular in the case of protopanaxatriol (PPT) derived ginsenosides, such as Re and Rg1. Expression of ginsenosides Rb1, Rb2, and Rc has been shown to be primarily controlled by environmental factors, while Rg1, Re, and Rd appear to be under at least partial genetic control[17,30]. Based on these observations Schlag and McIntosh[30] developed a system for grouping individuals into chemotypes based on the ratio of Re and Rg1: Re chemotype (RE), Re/Rg1 ≥ 2; intermediate (I), 1 < Re/Rg1 < 2; Rg1 chemotype (RG), 0 < Re/Rg1 ≤1. These chemotypes have been shown to correlate with genetic similarity, and are used here to define chemical profiles. Additionally, the ratio between ginsenosides Rg1/Rb1 is indicated as a diagnostic character in prior publications, and its usefulness as a descriptor of chemotypic diversity was examined. The demonstrated relationship between PPT ginsenoside profiles and genetic similarity offers the possibility of developing a deeper system of chemical fingerprinting to be used in differentiation of indigenous populations from those demonstrating introgression by commercial stock. Due to ginseng's history of cultivation in the region, such a system would allow ecologists as well as pharmaceutical companies to identify regionally native ginseng without relying on genetic analysis.



Ginsenoside	R_1	R_2
Rb1	-Glc^2-Glc	-Glc^6-Glc
Rb2	-Glc^2-Glc	-Glc^6-Ara(p)
Rc	-Glc^2-Glc	-Glc^6-Ara(f)
Rd	-Glc^2-Glc	-Glc

Ginsenoside	R_1	R_2
Re	-Glc^2-Rha	-Glc
Rg1	-Glc	-Glc

Figure 1. Chemical structure of six major ginsenosides.

Figure 2. Chemical structure of six major ginsenosides quantified in this study; (a) 20(S)-protopanaxadiol (PPD) derived species and (b) 20(S)-protopanaxatriol (PPT) derived species. Sugar moieties are represented as follows: Glc, glucose; Ara(p), arabinose (pyranose form); Ara(f), arabinose (furanose form); Rha, rhamnose. Adapted from Schlag & McIntosh 2006[28].

Historical patterns of overharvesting wild populations have been demonstrated to have reduced their fitness and diversity. Genetic diversity among wild populations is similar to the diversity found within cultivated populations, indicating low gene flow between wild populations[2,28]. without the effects of poaching, ginseng has a number of characteristics that predispose populations to low gene flow and tend to spatially segregate the genetic diversity of wild *P. quinquefolius*[13,29]. Such characters include relatively slow growth and maturation, high rates of self-pollination, high seed mortality, low dispersal rates, virtually nonexistent seed bank, and reliance on mature woodland habitat[1,7,12,22,33]. Partitioning of genetic diversity, and thus diversity of ginsenoside profiles, in populations of the Appalachian region is of particular interest due to the presence of a Tertiary relictual flora in the region[21]. Continental

drift occurring over the Paleogene and Neogene periods, and cycles of Quaternary glaciation lead to the formation of glacial refugia in the Southern Appalachian Mountains during the Pleistocene epoch. These refugia served as reservoirs of the biodiversity of the late Cretaceous period. In this study we hypothesized that Southern Appalachian populations would have distinct chemical signatures, with high diversity among populations. We also expected to find that populations were dominated by individuals with the RG chemotype, based on prior studies that have found primarily RG individuals in regions of the range of *P. quinquefolius* that were not glaciated during the Pleistocene.

2. Methodology

2.1. Sample Collection

Root samples from nineteen *P. quinquefolius* populations (Buncombe [7 populations], Haywood [1], Jackson [5], Macon [3], and Madison [1] counties in western North Carolina; Caroline County [2] in northeastern Virginia) were collected during the autumns of 2015 and 2016, using a partial root harvest methodology[27]. These comprise 7 public unprotected, 7 public protected, and 5 private sites. Ten percent of the large (3+ leaves), reproductive plants at each site were randomly selected for harvest, yielding 179 root samples. Single leaflets were collected for genetic analyses, and stored in vials filled with silica desiccant at -80 °C until processed. Root samples were dried for approximately 150 hrs at 35 °C, and ground through a 40 mesh screen on a Wiley Mini-Mill (Thomas Scientific, Swedesboro, NJ). Soil cores were collected within half a meter of plants for a subset of 67 individuals from 9 populations. These were analyzed at the North Carolina Department of Agriculture and Consumer Services soil-testing lab (http://www.ncagr.gov/agronomi/sthome.htm), and for carbon and nitrogen content by the NC State Environmental and Agricultural Testing Service (https://eats.wordpress.ncsu.edu/testing-services/).

2.2. Methanol-Reflux Extraction

Ginsenosides were extracted from dried roots via two one-hour MeOH-reflux extractions, using methods adapted from Corbit et al.[9]. One hundred milligrams dry, powdered root were heated to approximately 65°C in 5 mL of MeOH for one hour, and the solution was filtered through Grade 1 filter paper (Whatman). This process was repeated and the resulting filtrates, combined, were diluted to 20 mL and filtered through a 0.45 µm PVDF syringe filter (Thermo Scientific). Samples were stored at -20°C until analysis.

2.3. HPLC with UV Detection

Standard solutions of ginsenosides (Rg1, Re, Rb1, Rb2, Rc, and Rd) were prepared using pure ginsenosides (Indofine Chemical Company). Root samples and ginsenoside standards were analyzed with methods adapted from Court et al.[10]. Ginsenosides were separated by injecting 20 µL of each sample onto a Hypersil GOLD column (C_{18} column, 150 x 3 mm, 3 µm particle size) (Thermo Scientific) on a Prominence HPLC system (Shimadzu Inc., Columbia, MD) using gradient elution (H_2O/CH_3CN: 0-22 min, 95/5; 22-40 min, 78/22; 40-50 min, 55/45; 50-52 min, 45/55; 52-58 min, 35/65) with a flow rate of 0.6 mL/min. Column temperature was held at 35°C and absorbance was monitored at 205 nm. Retention time for each ginsenoside was determined from standards, and concentration was calculated using peak area on a chromatogram (Fig. 2), and a six-point external standard calibration curve.

2.4. DNA Extraction and Quantification of Microsatellites

Leaflets were harvested in the summers of 2014-2018 from 11 populations, including one Virginia population. The Ecological Plant Genetics lab at the University of North Carolina at Asheville processed samples and data. DNA was extracted using Qiagen DNEasy Plant MiniKit™. PCR was completed for 7 microsatellite loci described by Young et al.[38] : B011, B119, C009, C105, C202, D134, and D227. Multiplexed PCR samples were sent to the DNA Analysis Facility on Science Hill (Yale University) for fragment analysis. Peak data were analyzed in Geneious© version 11.2.

2.5. Statistical Analysis

Because many soil nutrient characteristics are likely autocorrelated, a Principal component analysis (PCA) was used to extract main components of variability in soil parameters[20] (PC-ORD; McCune and Medford 2011) (Table 1). Extracted PCA axis scores for each sample were used as synthetic variables in linear regressions in comparing root ginsenoside profiles. Euclidean distance matrices of ginsenoside concentrations, genetic distance and geographic distance were analyzed for correlation with a Mantel test[15,19] using *vegan* and *polysat* packages in R[5,8,24,40]. ANOVAs with Tukey's HSD post-hoc were used to identify significant differences in ginsenoside content between years and among populations (Fig. 4 & 5). An additional ANOVA was run to check for significant variation of ginsenosides by year of harvest. Two populations (DF and P1) were omitted from ANOVAs due to insufficient replication ($n<3$).

3. Results

Most populations in this study exhibited only the RG chemotype ($Re/Rg1<1$), and only 4 out of 19 populations displayed variation in chemotypes (Fig. 3). Population MC in particular contained individuals of all three chemotypes used, and accounted for the majority of RE and I chemotypes observed across North Carolina populations. Populations from Virginia exhibited distinct chemotypes, with VA1 containing mostly RG, while VA2 contained only RE individuals.

Mantel tests demonstrated significant positive correlation between the site of the plants and concentrations of ginsenosides Rg1, Re, and Rb1, as well as total concentration and Re/Rg1 ratio (Table 1, $p<0.05$, $n=173$). A second Mantel test excluding Virginia populations (VA1 and VA2) found the same relationships to be significant. A significant correlation was shown between genetic distance, based on 7 intergenic microsatellite loci, and the Rg1/Rb1 ratio (Table 2, $p=0.01$, $n=70$).

Principal component axes with eigenvalues greater than 1 were used for further analysis (Table 3). This included the first four axes, which cumulatively explained 80.04 % of the variance in soil characters. Multiple linear regressions were used to compare ginsenoside concentrations to the first four principal component axes of soil characteristics (Table 4). Axis 1 is associated with ion availability, and is strongly driven by increases in pH, base saturation (BS), cation exchange capacity (CEC), and concentrations of P, K, Ca, Mg, and Cu, and S. Axis 2 is driven by gross soil characters and macronutrients; bulk density, and % nitrogen and carbon, as well as acidity. Percent humic matter, with contributions from bulk density and several ion species primarily drive axis 3. Significant relationships were found between Axis 1 and Rg1, Axis 2 and Rg1, Re, Rd, and the two ratios of interest (Re/Rg1 and Rg1/Rb1), Axis 3 and Rg1, Re, Rb1, Rc, the ratios of interest, and total ginsenoside content (Table 4).

Analyses of variance (ANOVAs) comparing total ginsenosides, Re/Rg1 ratio, and Rg1/Rb1 ratio by site were used to assess variance in these composite characters (Figures 4 & 5). A Tukey's HSD test was applied post-hoc to identify significantly different values ($\alpha=0.05$). The Re/Rg1 ratio only significantly varied between population VA2 and all other populations, and is not shown. Values greater than plus or minus 2.2 times the interquartile range of the population were considered outliers.

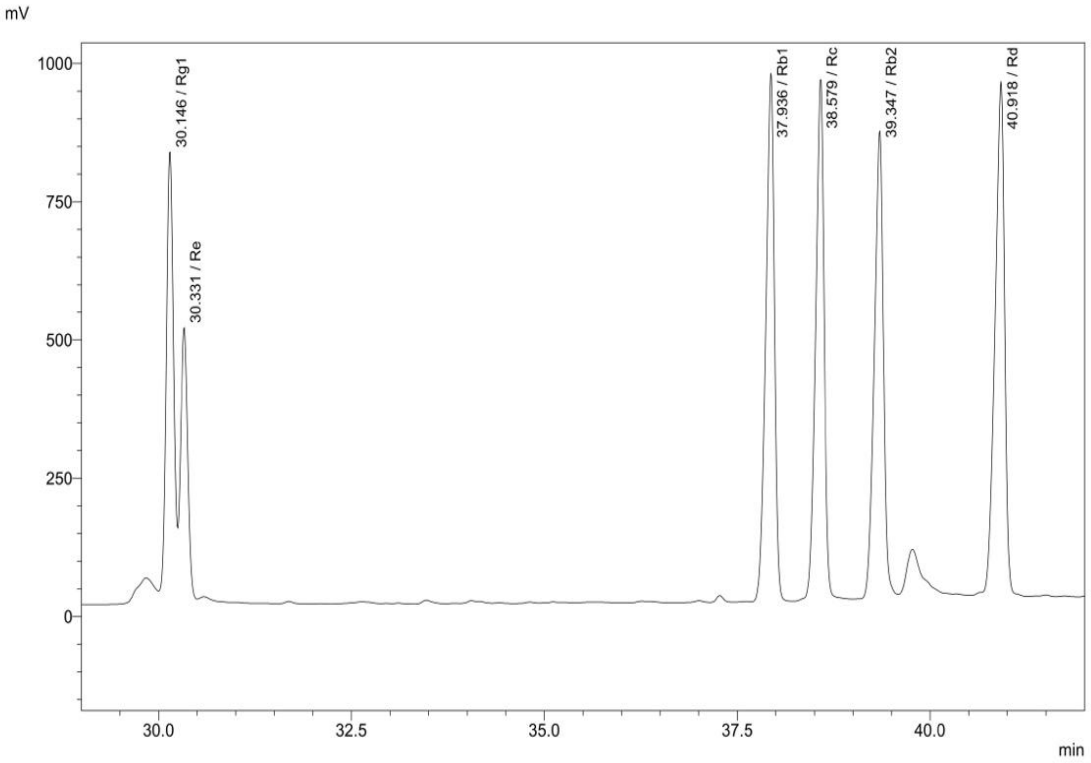


Figure 2. Example chromatogram of 167 ppm ginsenoside sample

Figure 2. Example chromatogram of 167 ppm ginsenoside sample. Absorbance peaks associated with the six ginsenosides studied (Rg1, Re, Rb1, Rc, Rb2, Rd) and retention times are shown.

Table 1. Mantel test results comparing chemical and geographic distance (Pearson's product-moment correlation, 100,000 permutations, n=173). Euclidean distance matrices were generated using ginsenoside concentration (mg ginsenoside/g dried root mass), and geographic coordinates. Significant p-values are indicated in bold.

	Rg1	Re	Rb1	Rc	Rb2	Rd	Total	Re/Rg1	Rg1/Rb1
Correlation coefficient	0.22030	0.37120	0.27040	0.05858	-0.00408	-0.02724	0.12260	0.44850	0.1645
p	**0.00030**	**0.00001**	**0.00003**	0.11696	0.47851	0.61447	**0.01031**	**0.00001**	**0.04813**

Table 2. Mantel test results comparing chemical and genetic distance (Spearman's rank correlation rho, 9999 permutations, n=70). Euclidean distance matrices were generated using ginsenoside concentration (mg ginsenoside/g dried root mass) and data on 7 microsatellite loci. Significant p-values are indicated in bold.

	Total	Re/Rg1	Rg1/Rb1
Correlation coefficient	-0.008173	0.07983	0.09845
p	0.5395	0.1476	**0.049**

Table 3. Principal component analysis (PCA) axes generated from soil composition at 67 individual plants from 9 sites in western North Carolina and the coastal plain of Virginia. PCA axes with eigenvalues < 1 were not included. Eigenvectors for each soil component are presented. Higher magnitude values (±0.6) indicate a stronger influence on the axis.

Parameters	**PCA 1**	**PCA 2**	**PCA 3**	**PCA 4**
Eigenvalue	5.853	4.744	1.977	1.033
Variance explained (%)	34.430	27.906	11.628	6.079
Cumulative variance explained (%)	34.430	62.336	73.964	80.043
C %	-0.0733	**-0.9216**	0.1326	-0.0642
N %	-0.0308	**-0.9202**	0.0838	-0.0253
Humic matter %	-0.0993	-0.4725	**-0.6912**	0.0558
Bulk (g/cm^3)	0.0644	**0.7696**	0.5216	-0.0946
pH	**0.6532**	0.0643	-0.0483	0.2618
Base saturation	**0.9331**	0.1238	0.2075	-0.0535
Ac (meq/100 g)	**-0.6959**	**-0.6405**	-0.2223	0.0079
Cation exchange capacity	**0.8251**	-0.4673	-0.0926	-0.1430
P (mg/dm^3)	0.4145	-0.3443	0.4538	-0.2558
K (mg/dm^3)	**0.7880**	-0.1982	0.0344	-0.2527
Ca (mg/dm^3)	**0.8951**	-0.2815	-0.0175	-0.1496
Mg (mg/dm^3)	**0.8478**	-0.2534	-0.1693	0.0279
S (mg/dm^3)	**-0.7568**	-0.3093	0.0106	-0.1321
Na (mg/dm^3)	-0.1844	**-0.7665**	0.3050	0.1267
Mn (mg/dm^3)	0.2036	-0.1380	0.4323	**0.8060**
Cu (mg/dm^3)	**0.6202**	-0.1753	-0.5182	0.2983
Zn (mg/dm^3)	-0.1581	**-0.7417**	0.5606	0.0117

Table 4. Results of linear regressions analysis of ginsenoside content use the first four principal component axes of a PCA of soil characteristics (α=0.05).

Ginsenoside		**Axis 1**	**2**	**3**	**4**
Rg1	R^2	0.06926	0.23650	0.05887	0.01772
	t-stat	-2.18	-4.45	-2.00	1.08
	p	**0.033**	**0.000**	**0.050**	0.287
Re		0.00004	0.24860	0.36070	0.01293
		-0.05	4.60	6.01	-0.92
		0.962	**0.000**	**0.000**	0.363
Rb1		0.02380	0.00209	0.10530	0.00728
		-0.95	0.37	2.75	-0.69
		0.348	0.715	**0.008**	0.496
Rb2		0.00547	0.00286	0.04995	0.01438
		-0.59	-0.43	1.83	-0.97
		0.555	0.670	0.071	0.338
Rc		0.01706	0.00408	0.06817	0.02866
		-1.05	-0.51	2.16	-1.37
		0.296	0.610	**0.034**	0.174
Rd		0.02692	0.06738	0.02483	0.00910
		-1.33	-2.15	-1.28	0.77
		0.188	**0.035**	0.206	0.446
Re/Rg1		0.00008	0.12650	0.15560	0.00271
		0.07	3.04	3.44	-0.42
		0.945	**0.003**	**0.001**	0.678
Rg1/Rb1		0.02364	0.10990	0.18500	0.03244
		-1.25	-2.81	-3.81	1.47
		0.218	**0.007**	**0.000**	0.148
Total		0.04191	0.00572	0.08327	0.00502
		-1.67	-0.61	2.41	-0.57
		0.099	0.546	**0.019**	0.572

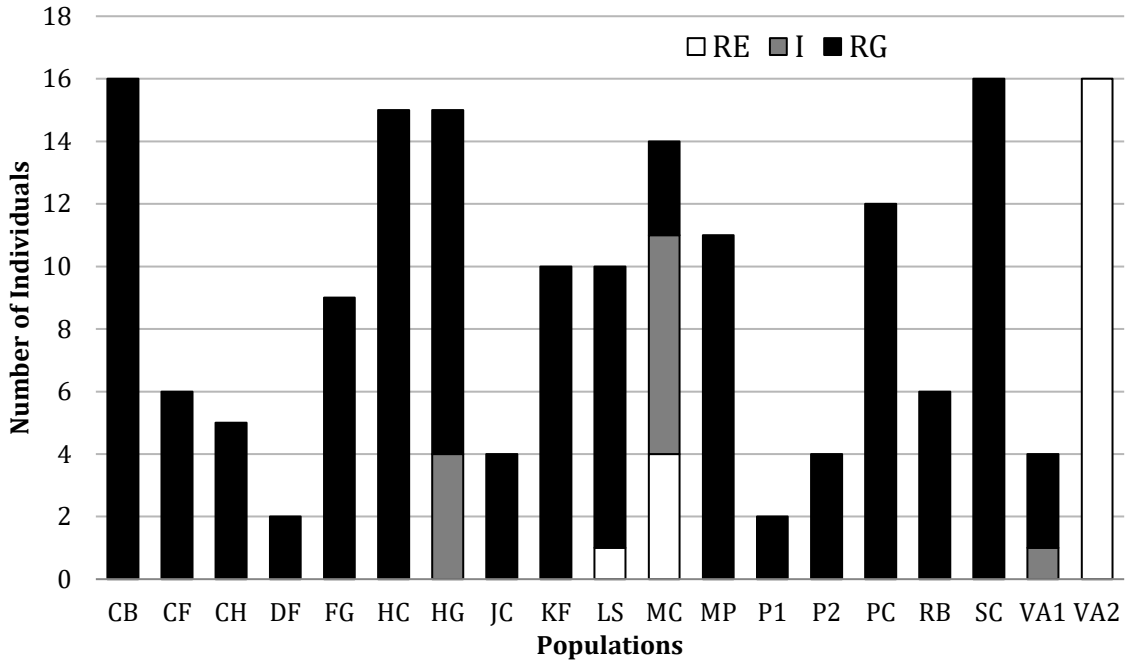


Figure 3. Chemotype of sampled individuals across all populations.

Figure 3. Chemotype of sampled individuals across all populations. Chemotype is based on the ratio of ginsenosides Re/Rg1, where an individual is classified as RE if Re/Rg1≥2, I if 1<Re/Rg1<2 and RG is o<Re/Rg1≤1.

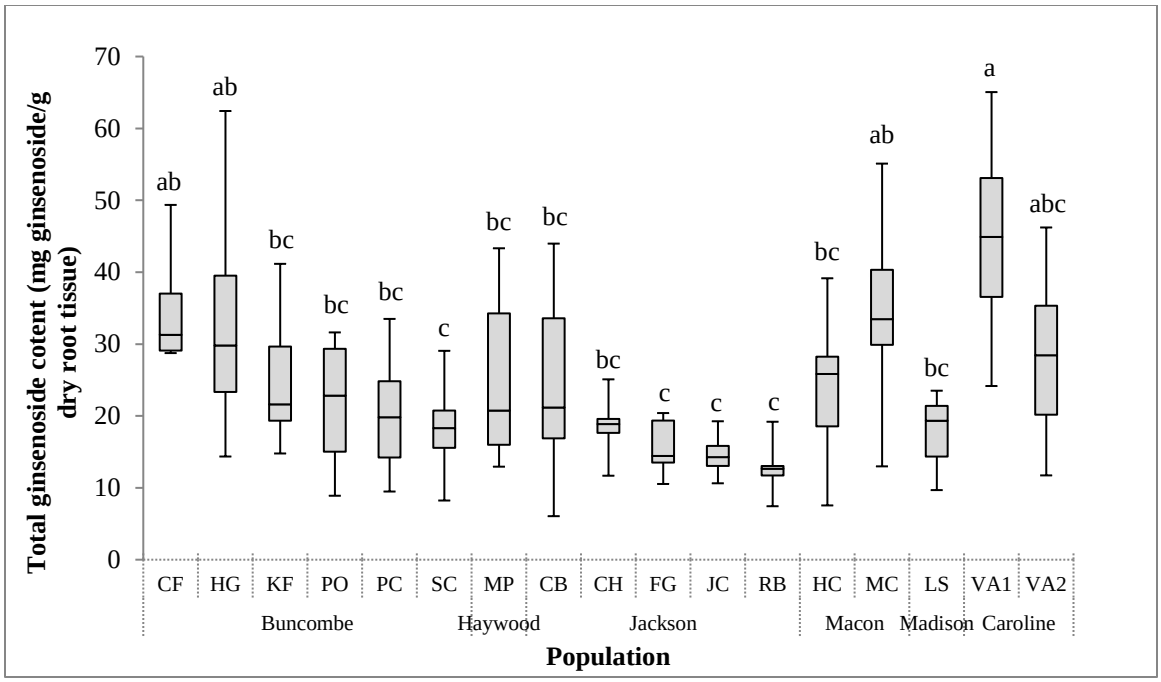


Figure 4. Boxplot summary of total ginsenoside content by population.

Figure 4. Boxplot summary of total ginsenoside content by population. Populations are grouped by county. An ANOVA coupled with Tukey's HSD post-hoc were used to determine significantly different means. Sites sharing a letter are not significantly different (α=0.05).

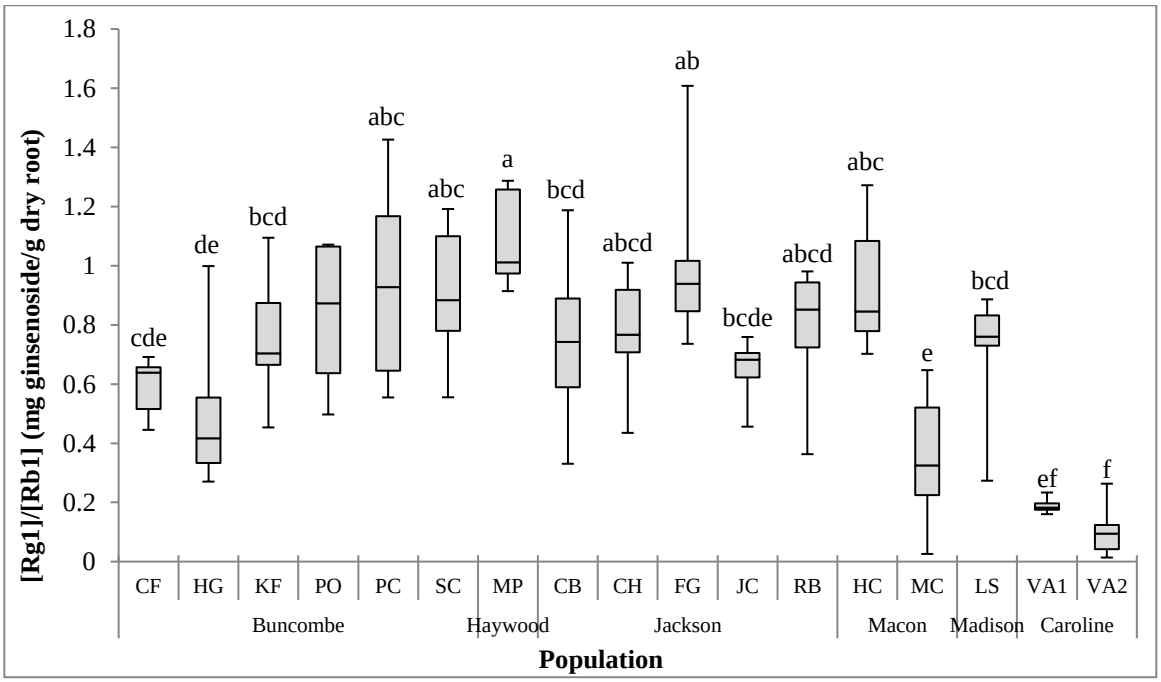


Figure 5. Boxplot summary of Rg1/Rb1 ratio by population.

Figure 5. Boxplot summary of Re/Rg1 ratio by population. Populations are grouped by county. An ANOVA coupled with Tukey's HSD post-hoc were used to determine significantly different means. Sites sharing a letter are not significantly different (α=0.05).

4. Discussion

Past studies of the ginsenoside profiles of American compared to Korean ginseng have found the ratio of Re/Rg1 to be greater than one for *P. quinquefolius* and less than one for *P. ginseng*, and the Rg1/Rb1 ratio to be less than 1/5 for *P. quinquefolius* and greater than 1/5 for *P. ginseng*[26]. The present study supports findings that neither of these demarcations hold true for all populations of *P. quinquefolius*. Most of the populations sampled in this study were found to contain exclusively individuals with a high Rg1-low Re chemotype (RG), and RG individuals were found in all populations. Additionally, nearly two-thirds (64.2%) of all samples contained only minute concentrations of Re (<0.01 mg ginsenoside/g dried root), similar to findings by Lim et al.[17] and Schlag and McIntosh[30]. The chemotype system used may need to be rethought for populations exhibiting concentrations of Re below the limit of detection, in particular when considering the consensus that concentrations of Re are strongly correlated with genetic similarity.

While the ANOVA comparing total ginsenoside content by population does show some significantly different groups, it is notable that population VA2, which was a chemotypic outlier, does not differ significantly from any other population. This indicates that total ginsenoside content is not a good measure of chemical similarity. The ANOVA comparing the Rg1/Rb1 ratio resolved many more significant relationships than the Re/Rg1 ratio, and grouped the two Virginia populations, as well as VA1 with another chemotypic outlier, population MC (Fig. 5). Integration of the Rg1/Rb1 ratio in future chemotyping schema may assist efforts to resolve the factors playing into ginsenoside expression. The significant correlation between the Rg1/Rb1 ratio and both geographic location and genetic markers indicates that there is a genetic basis for the different chemotypes seen in North Carolina populations when compared to prior studies. However the small correlation between the two variables (R^2=0.09845) suggests that genetic similarity is not the only factor influencing the observed ginsenoside concentrations.

The diversity of chemotypes within population MC, as well as the site's presence adjacent to a homestead, indicates that the population may contain introduced genes, particularly in comparison to the homogeneity of chemotypes found in other populations. MC was not significantly different from other populations in terms of Re/Rg1, but was significantly different from most in terms of Rg1/Rb1, including geographically proximal populations (Table 5). Again, this indicates the need for greater resolution of genetic differences to allow for more refined analysis of chemical diversity in the context of potentially introduced genetic stock.

Prior analysis has found concentrations of Rb1, Rb2 and Rc to be primarily controlled by environment, with Rg1 and Rd exhibiting limited influence by environmental factors, and Re showing none[17]. This study founds environmental correlates with all ginsenoside species other than Rb2 (Table 4). These data considered with the lack of genetic correlation, and presence of geographic correlation for Re, Rb1 and total ginsenosides indicate that non-soil environmental factors are driving the variation among populations. Additionally, concentrations of Rb1 (R^2=0.10530, t-stat=2.75, p=0.008) and Rc (R^2=0.06817, t-stat=2.16, p=0.034) have only limited linear correlation with PCA axis 3. These data contradict prior findings that production of these two ginsenosides is predominantly controlled by environmental conditions[17]. The limited or absent correlation of ginsenosides Rb1, Rb2 and Rc with geographic distances indicates that further analyses are required to resolve the differences that influence production of these species. Further examination of the factors controlling production could help integrate Rb1 and the Rg1/Rb1 ratio into future chemotyping schemes.

Understanding the year-to-year variation in chemical profile of individual plants could allow inclusion of control for such variation in future analyses. In particular, seasonal variation of ginsenoside concentrations in different plant organs could be introducing noise into these analyses. For this study, an ANOVA showed no significant variation of ginsenosides by year of harvest (p>0.1).

This study will inform further study of the interactions between environment and genetic structure in ginsenoside profiles. A common garden of plants from each population has been established in western North Carolina with paired plants from four of the populations sampled, and may help resolve whether that relationship is the result of population structuring, such as disjunction of populations in glacial refugia, or direct environmental factors. Existing data on photosynthetic rates may also prove useful in controlling for differences in plant health and vigor. Additionally an analysis for correlation between ginsenoside concentrations and individual microsatellite loci may prove useful, since the loci are noncoding regions of the genome and may be associated to various degrees with genetic mechanisms of regulating expression.

The therapeutic benefits of ginseng are contingent upon the extremely diverse physiological effects and interactions of ginsenosides, including reported activities in the central nervous, immune, and vascular systems[3,4,16,25,31,37,39]. While *P. quinquefolius* is often characterized as an adaptogen, or cure-all, our findings suggest that plants from different populations could possess different medicinal properties based on geographic and genetic origins. These preliminary results support our hypothesis and suggest that even within the limited distribution of our study there are distinct ginsenoside profiles. Refinement of chemotype classification, with inclusion of genotypic and phenotypic correlates, will assist efforts to discriminate between native and introduced populations, and will facilitate cultivation of specific ginsenoside profiles for medicinal use.

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