

Abstract

Title of Thesis: **GENETIC DIVERSITY AND
PHYTOCHEMISTRY OF MARYLAND-
GROWN AMERICAN GINSENG (*PANAX
QUINQUEFOLIUS* L.)**

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American ginseng (*Panax quinquefolius*) is a valuable medicinal herb threatened by over-harvest. Because data are insufficient, current attempts to protect diversity and improve cultivation are inadequate. Random Amplified Polymorphic DNA (RAPD) markers were used to estimate genetic diversity and high-performance liquid chromatography (HPLC) was used to characterize root ginsenoside concentrations in wild and cultivated populations of American ginseng in Maryland. Wild populations were less diverse than cultivated and highly differentiated from one another; suggesting that drift was high and gene flow low in wild populations. Exotic plants were genetically and phytochemically distinct from native plants. The main ginsenoside in exotic plants was Re versus Rg1 in native plants. Plants from at least one wild population were genetically and phytochemically similar to exotic plants, suggesting that exotic plants were introduced into wild populations. Thus, native Maryland American ginseng is unique but threatened by drift, isolation and artificial introductions.

GENETIC DIVERSITY AND PHYTOCHEMISTRY OF MARYLAND-GROWN
AMERICAN GINSENG (*PANAX QUINQUEFOLIUS* L.)

By

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Dedication

I would like to dedicate this thesis to my family, who provided me with an escape from my research when needed and left me refreshed and ready to work again; to my husband who was always willing to listen, be interested and provide valuable insight into my research and who loved and supported me through good research times and bad; to all of my academic mentors, including my thesis committee: Dr. Maile Neel, Dr. José Costa and Dr. Theophanes Solomos; my supervisors at N.I.H.: Dr. David Wassarman and Dr. Lori Pile and my undergraduate advisor Dr. Ron Hoham, for inspiring me to continue my education and do solid research that answers important questions; and finally to my thesis advisor, Dr. Marla McIntosh, for always providing fresh, new perspectives, challenging me to work and think independently, and for being available and ready to help when difficulties and questions arose.

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Chapter 1: Literature Review

GEOGRAPHIC DISTRIBUTION AND HABITAT

American ginseng (*Panax quinquefolius* L.) is a perennial herb in the family Araliaceae that is native to the eastern hardwood forests of the United States and Canada. The natural range of American ginseng extends from Quebec and Ontario, west to Kansas and south along the Appalachian Mountain range into Georgia and Louisiana (USDA NRCS, 2002). American ginseng grows on well-drained soils (Anderson et al., 1993), achieving maximum growth at 8 to 30% of full sunlight (Park and Lee, 1993; Proctor and Bailey, 1980) and tolerates a wide range of soil fertility and pH conditions (reviewed in Anderson et al., 2002). To enhance survival, roots may form mycorrhizal associations (Anderson et al., 1993; Carpenter and Cottam, 1982). Based on a niche analysis of known American ginseng habitats, American ginseng is most common in forests on north-facing slopes, at middle to low elevations and in sites protected from direct solar radiation such as narrow ravines or coves (Anderson et al., 1993). However, a recent survey using stratified random sampling of forestlands in West Virginia found the greatest abundance of ginseng on east-facing slopes and west-facing slopes at middle to low elevations (McGraw et al., 2003). It was suggested that these 'suboptimal' east- and west-facing forests might act as refugia from harvesters.

SYSTEMATICS

Many plant species are commonly referred to and sold as ginseng. The most commonly marketed medicinal herbals sold as ginseng are: Asian (Korean or

Chinese) ginseng (*Panax ginseng* C.A. Meyer) and American ginseng (*P. quinquefolius*). Another phytomedicinal in the Araliaceae family, Siberian ginseng (*Eleutherococcus senticosus*), was also sold as ginseng until the FDA limited ginseng sales to *Panax* species in the U.S. Other less valuable ginseng species include: Sanchi ginseng (*Panax notoginseng* Burk), Japanese ginseng (*Panax japonicus* C.A. Meyer), Vietnamese ginseng (*Panax vietnamensis* Ha et Grushv) and various subspecies of *Panax psuedoginseng* (Shibata, 2001). Dwarf ginseng, *Panax trifolius* L., like *P. quinquefolius* is native to North America; however, *P. trifolius* roots are small and are not used as an herbal remedy. The two primary ginseng herbs, *P. quinquefolius* and *P. ginseng*, are tetraploid ($2N = 4x = 48$) species (Duke, 1984; Mathur et al., 2003). These species are believed to be allopolyploids with disomic rather than tetrasomic inheritance (Duke, 1984). According to a study of the phylogeny of *Panax* and *Aralia* species (Wen and Zimmer, 1996), tetraploid *Panax* species fall within clades of equal or lesser age than diploid species indicating that these tetraploids evolved from more primitive diploid species. Evidence for two connections between East Asian and North American disjunct *Panax* species was found. The North American species *P. quinquefolius* and *P. trifolius* were distinct from each other and *P. quinquefolius* was more closely related to the advanced Asiatic species (including *P. ginseng*).

HARVEST HISTORY

Father Joseph Francis, a French Jesuit missionary living among the Iroquois Indians in North America, discovered American ginseng (*P. quinquefolius*) near Montreal, Canada in 1716 (Persons, 1994). Father Francis identified American

ginseng based on descriptions of Asian ginseng (*P. ginseng*), an herbal panacea or “cure-all” that had been used for thousands of years in Chinese medicine (Persons, 1994). By the 1700s wild Asian ginseng populations had suffered the effects of severe over-harvest and American ginseng was immediately exported to Asia to satisfy the demand for wild ginseng (Persons, 1994). Cultivation of American ginseng in North America began in the late 1800s, by which time wild American ginseng roots had become increasingly difficult to find (Anderson et al., 2002). However, cultivation of American ginseng is difficult and cultivated roots are much less valuable than wild roots. The Asian grading system is based in part on the visual appearance of the roots, which is associated with method of production. Wild roots that are dark in color, gnarled and branched (Figure 1) are generally considered more medicinally potent than cultivated roots which are typically light in color and consist of a single, straight taproot (Beyfuss, 1999).

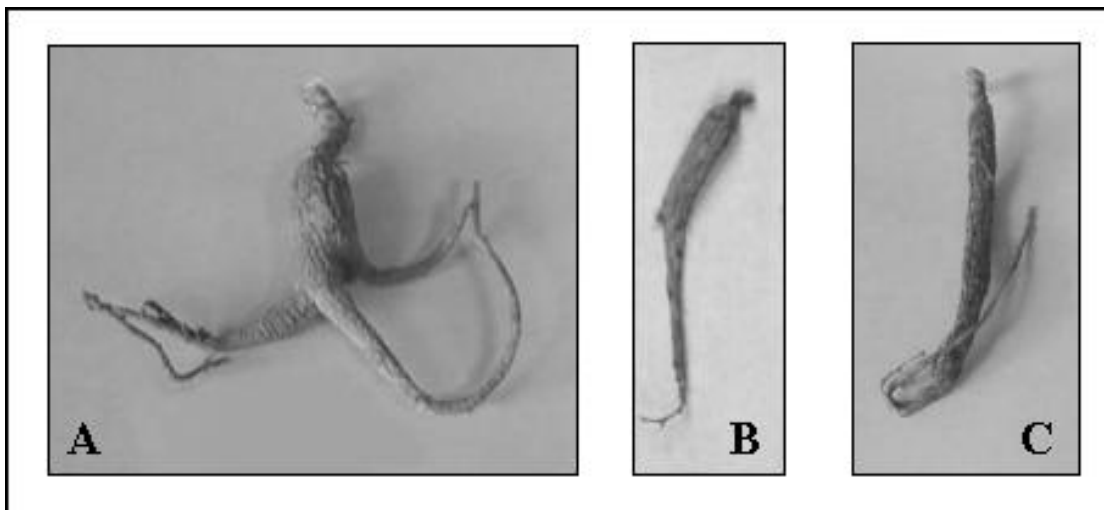


Figure 1. Images of three dried American ginseng roots with variable branching patterns (not to scale): (A) wild-simulated root (4.11 g dry weight); (B) wild-simulated root (1.89 g dry weight); (C) wild root (1.43 g dry weight).

Wild-simulated or woods-grown roots are cultivated under more natural forest conditions. Stratified ginseng seed is planted in an appropriate forested site (considering light irradiance, pH, calcium levels, soil type and drainage) and grown until it reaches an acceptable harvest size (6-10 yrs). If allowed to grow naturally without tilling, these roots will strongly resemble a wild root, making them much more valuable than traditionally cultivated plants (Figure 1).

Ginseng (*Panax* species) remains one of the most revered medicinal plants in traditional Chinese medicine and is quickly becoming one of the most popular medicinal herbs in the Western market, consistently ranking in the top three in retail sales in North America (Hall et al., 2001). Sales of ginseng supplements exceed \$300 million annually in the U.S. (Court, 2000). The U.S. is the chief supplier of wild American ginseng to overseas markets and approximately two-thirds of the harvest is exported to China (Robbins, 1998). Although the market price for wild roots has consistently been much greater per kg than for cultivated roots, most American ginseng exported currently from the U.S. is cultivated (total exports peaking at over one million kg in 1989 and 1994) (Chamberlain and Predny, 2002). Wholesale prices for wild roots have been volatile over the last 12 years, but wild roots have sold for as much as \$1000 per kg (dry weight) whereas cultivated roots have sold for as little as \$20 per kg, less than their production cost (Hankins, 2000). The demand for wild American ginseng has continued to rise over the past 100 years and exports of wild root from 1992-2001 averaged approximately 50,000 kilos per year with peak exports in 1996 (approximately 200,000 kg) (Chamberlain and Predny, 2002). In addition, there has been a general upward trend in number of roots per kg of dry ginseng

certified for export, suggesting that fewer mature ginseng plants are available for harvest (Robbins, 2000). Historically, American ginseng has provided significant revenue to rural communities. From 1983 to 2000, 100 thousand kg of wild ginseng was exported annually and generated revenue of \$71.7 million. While there is low demand and an over supply of cultivated American ginseng, the economic value of wild American ginseng is substantial.

LIFE HISTORY

Aboveground, American ginseng has a whorl of palmate-compound leaves located at the apex of a tall stem (Figure 2). Belowground it has a short stem (rhizome) connected to a thick, branched taproot that may resemble the shape of the human body (Figure 2). American ginseng is a perennial, herbaceous plant and the aboveground biomass dies back after each growing season. At the connection of the stem and rhizome a new bud is formed each year that produces a new shoot the following growing season. Thus, plant age can be estimated by counting the number of bud scars that result from annual stem abscission (Anderson et al., 1993). As American ginseng plants mature they increase in size and in number of leaves and leaflets. Juvenile plants produce a single leaf bearing three leaflets while reproductive plants may contain two to five leaves each bearing three to five leaflets (Carpenter and Cottam, 1982). Although stem height, root weight and number of leaves and leaflets can indicate plant age, variation in these relationships is common because of environmental variations, including differences in site characteristics, precipitation and temperature (Anderson et al., 1993; Carpenter and Cottam, 1982; Lewis and Zenger, 1982; Lewis and Zenger, 1983), and natural disturbances, most

notably white-tailed deer herbivory (Anderson et al., 2002). Wild ginseng grows slowly and typically reaches maturity around 6 years of age (Anderson et al., 2002; Lewis and Zenger, 1982), but plants grown in cultivated populations typically mature in as few as three years (Carpenter and Cottam, 1982).

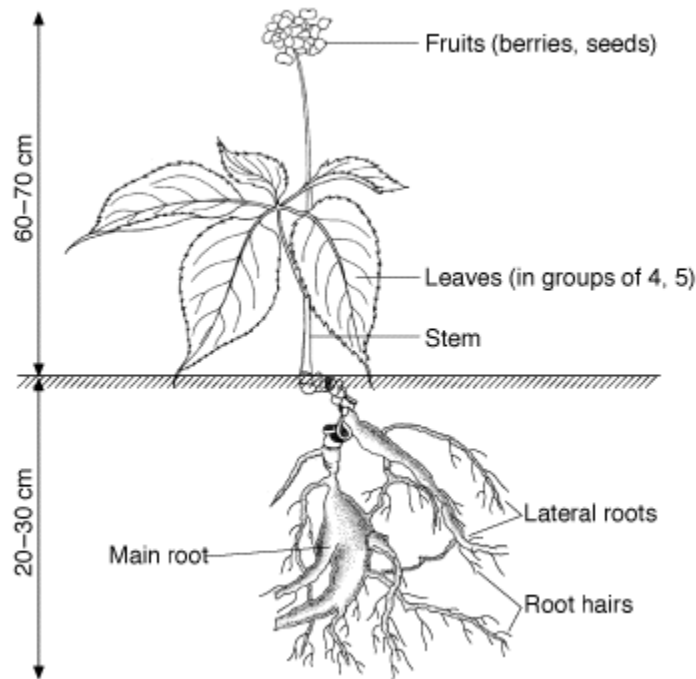


Figure 2. An illustration of a mature American ginseng plant. Content of figure is adapted from Sticher, 1998.

REPRODUCTIVE BIOLOGY

American ginseng is believed to rely almost exclusively on sexual reproduction (Lewis and Zenger, 1982; Van der Voort et al., 2003). Flowering begins in June and lasts approximately four weeks (Anderson et al., 1993; Schluter and Punja, 2000). Plants produce a solitary umbel and many small, perfect, greenish-white flowers. Flowers are bisexual and may exhibit protandry (Carpenter and

Cottam, 1982; Lewis and Zenger, 1983; Schlessman, 1985; Schluter and Punja, 2000) although a hermaphroditic stage (pollen release, stigmas receptive) has been detected in American ginseng flowers (Schlessman, 1985). Flowering is sequential in the umbel with approximately 10% of flowers releasing pollen at one time (Schluter and Punja, 2000). Both phenological (Schlessman, 1985) and genetic data (Schluter and Punja, 2002) suggest that American ginseng has a mixed-mating system which includes outcrossing (xenogamy) and self-pollination (autogamy and geitonogamy) (Anderson et al., 1993; Schlessman, 1985). However, the bagging of inflorescences can increase seed set, suggesting that American ginseng is highly self-compatible (Schluter and Punja, 2000). Emasculated flowers do not produce seed in bagged inflorescences, hence seed set is the result of self-pollination not apomixis (Carpenter and Cottam, 1982; Schlessman, 1985). Generalist pollinators of American ginseng include halactid bees (*Dialictus*), ants and syrphid flies (Lewis and Zenger, 1983; Schluter and Punja, 2000).

American ginseng fecundity is generally low (Lewis and Zenger, 1983) and may be regulated more by resource allocation than pollination success (Carpenter and Cottam, 1982; Schlessman, 1985) as larger plants typically produce greater numbers of flowers, fruits and seeds (Lewis and Zenger, 1982; Schlessman, 1985). Each flower can produce one to three seeds, which are enclosed in a pericarp that turns to a bright red upon ripening (Lewis and Zenger, 1983). Red fleshy fruits are often animal dispersed, but most ginseng fruits fall within a meter of the parent plant indicating that seeds are instead dispersed by gravity (Anderson et al., 2002). Seeds mature in August through September and are dormant for 18-22 months before

germination in the second spring (Anderson et al., 1993; Lewis and Zenger, 1982). It has been inferred that a seed bank may be present, but that the number of seeds is expected to be small and viability short (Charron and Gagnon, 1991; Lewis, 1988).

POPULATION DEMOGRAPHICS

American ginseng, like many forest perennial herbs, exhibits low recruitment and establishment rates and finite population growth rates close to 1.0 (Charron and Gagnon, 1991). American ginseng has been documented to live for more than 50 years (Charron and Gagnon, 1991); however, reports of plants older than 10 years are uncommon in natural sites (Anderson et al., 1993; Carpenter and Cottam, 1982; Lewis and Zenger, 1982). Using a projection matrix model of size classes, estimates of finite population growth rates in four natural populations of American ginseng observed in Canada ranged from 0.87 to 1.19 over three years (Charron and Gagnon, 1991). Elasticity analyses revealed that changes affecting the largest plants (three- and four-leaved) had the greatest impact on population growth (Charron and Gagnon, 1991) because they produced more seeds and because they had a higher probability of survival than smaller plants (Charron and Gagnon, 1991). In a single American ginseng population in Missouri, Lewis and Zenger (1982) found that seed mortality was high over the three years of the study, but once seedlings were established plant survivorship increased to 97%. However, adult survivorship is likely to be dependent on climate, site characteristics and other natural disturbances such as disease and herbivory. American ginseng is susceptible to at least a dozen pathogenic fungi such as *Alternaria panax*, *Phytophthora* species and *Fusarium* species (Persons, 1994). *A. panax* is particularly destructive and causes damping-off, blight and root rot (Persons,

1994). White-tailed deer have the potential to consume the entire inflorescence or aboveground portion of a plant and as deer populations grow the impact of herbivory is likely to increase.

IMPACTS OF HARVEST: DEMOGRAPHICS

Wild American ginseng has been declining across its range primarily due to habitat destruction (deforestation) and over-harvesting of plants. Harvest pressure has contributed to declines in: population size (Lewis, 1988; McGraw et al., 2003; Van der Voort et al., 2003), plant size (McGraw, 2001; Robbins, 2000), the proportion of larger, older plants (Anderson et al., 2002; Anderson et al., 1993; Cruse-Sanders and Hamrick, 2004) and finite population growth rates to below 1.0 (Charron and Gagnon, 1991; Lewis and Zenger, 1982; Nantel et al., 1996; Van der Voort et al., 2003). Wild American ginseng populations are generally thought to be small. A recent survey of wild American ginseng populations located mainly in West Virginia confirmed that populations are widespread, but sparsely populated (McGraw et al., 2003). Populations ranging in size from fewer than 10 to over 1,000 individuals have been reported in the literature (Cruse-Sanders and Hamrick, 2004; Grubbs and Case, 2004; Van der Voort et al., 2003); however, actual survey data are meager. In one survey of wild American ginseng in West Virginia, populations generally contained less than 100 individuals, often fewer than 10 (McGraw et al., 2003). Also, McGraw et al. (2003) found that herbarium specimens of American ginseng collected in Midwestern, Appalachian and Southern states showed sharp declines in stature over the past 150 years, the most likely cause being selective harvest of larger plants. Roots being exported to Asia also appear to be smaller than

previously collected (Robbins, 2000). In 1995, Maryland dealers reported a higher average number of roots per kg harvested (660-1100 dry roots per kg) than any other state listed (Robbins, 2000), suggesting that wild roots were smaller in Maryland than in other states (given that it is unlikely that harvesters preferentially harvested small plants).

Age structure in wild populations of American ginseng has been shown to shift toward younger, smaller plants after harvest. Anderson et al. (1993) found marked decreases in the number of three-leafed (large) plants, and thus seed production, on unprotected sites in Illinois versus those protected from harvest. A severe harvest event was simulated in West Virginia, which left less than one half of the original number of plants on the site. After two years, stem number exceeded the pre-harvest count; however, the percent of reproductive individuals had only risen to 26% by the fifth year in comparison to 78% in the pre-harvest population (Van der Voort et al., 2003). Anderson et al. (2002) found that when populations were protected from harvest, the upper age limit of plants was approximately 27-30 years, but if harvest had occurred, very few plants were greater than 10 years old. Cruse-Sanders and Hamrick (2004) found that age class structure shifted toward smaller, non-reproductive plants in unprotected populations of American ginseng as compared to populations that were protected from harvest.

Charron and Gagnon (1991) observed the population dynamics of four American ginseng populations in southern Quebec for 3 years and simulated the effects of harvest on population survival. They found that a maximum of 16% of plants (collected equally across size classes) could be harvested in a good growing

season without threatening population survival. However, because growing conditions significantly affect American ginseng fecundity, no plants could be sustainably harvested in a poor growing season (Charron and Gagnon, 1991). Lewis and Zengler (1982) found that under adverse environmental conditions (dry and hot), fruit production of American ginseng plants was reduced 47%. Elasticity analyses revealed that loss of large plants had the greatest affect on finite population growth rate (Charron and Gagnon, 1991) but harvest simulations did not account for size-selective harvest. Because large, mature American ginseng plants are typically collected preferentially over small, juvenile plants, the sustainable harvest levels provided by Charron and Gagnon (1991) may be biased upward. Nantel et al. (1996) conducted a population viability analysis of the transition matrices published for the four populations in Quebec under various harvesting regimes and incorporating environmental stochasticity. Mean finite population growth rate was 1.04 and the maximum rate of sustainable harvest was 5% per year; three times lower than estimated for a stable environment (Charron and Gagnon, 1991). Nantel et al. (1996) estimated that the minimum viable population size was 172 plants (including reproductive and non-reproductive individuals) for American ginseng. Unfortunately populations of this size are rarely observed in nature (Lewis, 1988; McGraw et al., 2003; Van der Voort et al., 2003). Minimum viable population size was estimated based on simulations that did not incorporate size-selective harvest or inverse-density dependence (Allee effect); however in experimental American ginseng populations reproductive success was found to be lower in small populations (Hackney and McGraw, 2001). Given the existence of Allee effects in American ginseng

populations and size-selective harvest the maximum harvest rate suggested by Nantel et al. (1996) (5%) may also be biased upward. In general, only very low harvest rates appear to be sustainable although collectors can easily harvest the majority of plants upon a single visit to a site and more than one collector may visit a site within a growing season.

MEDICINAL PROPERTIES

American ginseng roots are used in traditional Chinese medicine to treat a large variety of ailments ranging from fatigue and stress to cancer (reviewed by Attele et al., 1999; Coleman et al., 2003; Court, 2000; Shibata, 2001; Sticher, 1998; Tanaka, 1994). Asian ginseng has been used as a general tonic to improve vitality and longevity for thousands of years (Persons, 1994). According to the ancient “Oriental Doctrine of Signatures”, the shape of the root confers its therapeutic value. Because the ginseng root has many branches that can form the shape of a human, it is thought to act as a panacea or “cure-all” that brings about well being by balancing the body’s yin (cold, dark, feminine) and yang (warm, light, male) forces (Court, 2000; Persons, 1994). In general, the pharmacological effects associated with ginseng use include “adaptogenic” effects or the recovery of homeostasis (maintenance of chemical and metabolic equilibrium), effects on the central nervous system (CNS) (stimulatory or sedative effects, improved memory and learning), cardiovascular effects (lowering blood pressure), antipsychotic effects, stress alleviation, improved gastrointestinal motility, immunological effects (most importantly the inhibition of tumor growth) and enhancement of sexual behavior (Sticher, 1998; Tanaka, 1994). The traditional use of ginseng as a general tonic to increase longevity (anti-aging) and

vitality is likely associated with ginseng's role in the maintenance or recovery of homeostasis, for example the lowering of cholesterol and blood sugar levels.

The pharmacological effects of ginseng have been attributed primarily to a class of dammarane-type tetracyclic triterpenoid saponins, also known as ginsenosides. Other bioactive compounds isolated from *Panax* species include: antioxidants, polyacetylenic alcohols, peptides, fatty acids, polysaccharides, vitamins (e.g. vitamin C, thiamine, riboflavin, vitamin B-12 and nicotinic acid) and minerals (e.g. manganese, copper, cobalt and arsenic) (Huang, 1999; Sticher, 1998). More than twenty ginsenosides have been isolated from ginseng roots and leaves and are classified into two main groups: the glycosides of 20(S)-protopanaxadiol (20[S]-dammar-24-ene-3 β , 12 β , 20-triol) (Rb1, Rb2, Rc, Rd, Rg3 and Rh2) and those of 20(S)-protopanaxatriol (6 α -hydroxy-20[S]-protopanaxadiol) (Re, Rf, Rg1, Rg2, Rh1 and R1) (Court, 2000; Tanaka, 1994) (Figure 3). Ginsenoside Ro, a minor component in ginseng is an oleanane-type pentacyclic triterpene (Shibata, 2001). Ginsenosides were named according to their polarity, which decreases from Ra to Rh (Sticher, 1998). Ginsenosides contain a four *trans*-ring rigid steroid skeleton with a modified side-chain at C-20 and differ structurally in the number and placement of sugar moieties and hydroxyl groups (reviewed by Attele et al., 1999). Ginsenosides are amphiphilic and may act as lipid-soluble signaling molecules in a similar manner to steroids (Attele et al., 1999). Ginsenosides may regulate gene expression through the binding of intracellular steroid receptors. Rg1 has been shown to be a functional ligand of the nuclear glucocorticoid receptor (Attele et al., 1999). Other possible mechanisms for activity include the binding of plasma membrane steroid receptors

that trigger a non-genomic signal or the binding of membrane receptors that trigger changes in electrolyte transport systems (Attele et al., 1999). The structural diversity and multiple targets of ginsenosides are likely to be responsible for the diversity of pharmacological effects documented for ginseng (Attele et al., 1999).

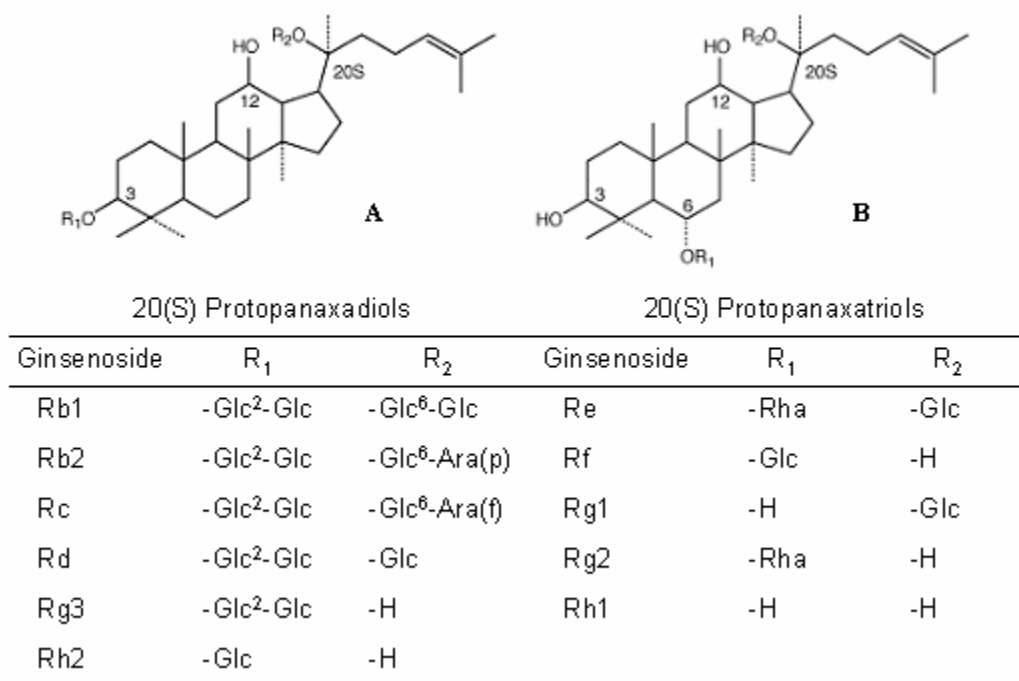


Figure 3. Chemical structures of two major groups of ginsenosides: (A) 20(S) Protopanaxadiols and (B) 20(S) Protopanaxatriols. Glc, glucose; Ara(p), arabinose in pyranose form; Ara(f), arabinose in furanose form; Rha, rhamnose; H, hydrogen. Content of figure is adapted from Attele et al. (1999) and Shibata (2001).

American ginseng and Asian ginseng are thought to differ in their medicinal properties. Whereas Asian ginseng is thought to have a stimulating effect, American ginseng is characterized as more soothing. The main ginsenoside constituents of each of these species, Rg1 (Asian ginseng) and Rb1 (American ginseng), have been found to have differing pharmacological effects. Reputed pharmacological effects of

ginsenoside Rg1 include weak central nervous system and motor activity stimulation, antifatigue action and aggravation of stress ulcers, whereas the reputed effects of Rb1 include central nervous system depression, antipsychotic action, increased gastrointestinal motility, anti-hemolytic and anti-stress actions (reviewed by Shibata, 2001). Rb1 and Re are thought to improve memory (Attele et al., 1999; Sticher, 1998) and Rg1 is thought to increase immune response (Attele et al., 1999) and cellular metabolism (Sticher, 1998). Thus ginsenoside composition appears to affect pharmacological activity.

GINSENSIDE VARIATION

Market value of ginseng in Asia is based on age, root color and shape, species and method of cultivation, which are believed to be indicative of chemical content and medicinal properties. Ginsenosides are unique to *Panax* species, have been associated with the pharmacological activities of *Panax* species and thus are used as marker compounds for quality control (Attele et al., 1999; Chuang et al., 1995; Huang, 1999). However, ginsenoside concentration varies widely in commercial samples (Harkey et al., 2001) and there is concern about the purity and potency of dietary supplements containing ginseng (Angell and Kassirer, 1998). As previously mentioned, American ginseng is noted for having higher concentrations of Rb1 and Re ginsenosides relative to Asian ginseng which has greater concentrations of Rg1 and Rf ginsenosides (Court, 2000; MoraMarco, 1997). Ginsenosides isolated from American ginseng include: Rb1, Rb2, Rc, Rd, Re, Rg1 and Ro (Court et al., 1996a; Court et al., 1996b; Li et al., 1996) and the main constituents extracted from cultivated American ginseng are Rb1 and Re (Li et al., 1996). American ginseng is

most easily distinguished from Asian ginseng by the absence of the ginsenoside Rf and less consistently by the ratio of Rg1:Rb1 (Assinewe et al., 2003; Chuang et al., 1995; Harkey et al., 2001). American ginseng is noted for being the only species to have Rg1:Rb1 ratios of less than 1.0 (~0.15); however, some wild roots have been analyzed that possess greater concentrations of Rg1 than cultivated roots, making this ratio an unreliable indicator of species (Assinewe et al., 2003; Attele et al., 1999; Chuang et al., 1995). Total ginsenoside concentration also varies among species and ranges from approximately 2-20% (Attele et al., 1999). Total ginsenoside is generally higher in American ginseng than Asian ginseng; however, *P. notoginseng* and *P. japonicus*, less commercially valuable species, have contained the highest total ginsenoside reported for ginseng species (Awang, 2000; Chuang et al., 1995; Huang, 1999).

A number of studies have been conducted that considered the effects of age and dry weight of roots on ginsenoside concentration in cultivated American ginseng plants (Court et al., 1996a; Fournier et al., 2003; Li and Mazza, 1999; Li and Wardle, 2002; Li et al., 1996; Park and Lee, 1993; Smith et al., 1996; Wills et al., 2002). In cultivated populations, age and root weight were directly correlated with total ginsenoside concentrations (Court et al., 1996a; Wills et al., 2002); however, these relationships are little studied or less clear in wild populations (Assinewe et al., 2003). In cultivated populations, root weight has been found to increase with age (Court et al., 1996a) and to be a good predictor of ginsenoside concentration (Smith et al., 1996; Wills et al., 2002). However, it is generally thought that wild roots, which are typically smaller than similarly aged cultivated roots, may be more potent than

cultivated roots (Chuang et al., 1995). Given the differing environmental conditions between wild and cultivated sites (e.g. soil fertility, exposure to pests) it is likely that the relationship between age, root weight and ginsenoside concentration may differ for wild and cultivated plants.

Ginsenoside concentrations have also been shown to vary by stage of plant development (Li and Mazza, 1999; Li and Wardle, 2002; Wills et al., 2002), plant tissue type (Assinewe et al., 2003; Li et al., 1996; Smith et al., 1996; Wills et al., 2002) and light levels (Fournier et al., 2003; Park and Lee, 1993). Wills et al. (2002) found that total ginsenoside concentration was greatest in the leaves and roots of American ginseng at initial fruit set. As the fruit matured, leaf ginsenosides increased, but root ginsenoside did not change. Other studies have agreed that leaf ginsenoside concentration increases with plant maturity (Li and Mazza, 1999; Li and Wardle, 2002); but reported that root ginsenoside decreased as the plant developed (Li and Wardle, 2002). Leaves, stems, and various root parts have been shown to differ in ginsenoside concentrations (Assinewe et al., 2003; Li et al., 1996; Smith et al., 1996; Wills et al., 2002). In cultivated populations, ginseng leaves had higher total ginsenoside concentrations (% w/w) than roots (leaf: 2.4-6.1%, root: 2.4-3.9%) (Li et al., 1996; Wills et al., 2002); however, in wild populations roots had higher total ginsenoside concentrations than leaves (leaf: 3.3%, root: 5.8%) (Assinewe et al., 2003). Perhaps age has a larger positive effect on root ginsenoside than leaf and therefore older wild roots have higher ginsenoside concentrations than leaves. Regardless, the concentration of ginsenoside in leaves is appreciable and could provide an alternative source of ginsenoside for herbals. Soil nutrient status of

nitrogen, calcium, magnesium and phosphorus in the soil, are not good predictors of root ginsenoside concentration, but may be better indicators of leaf ginsenoside concentration (Li et al., 1996). However, slightly acidic soils with high levels of iron and sulfur may produce ginseng with higher ginsenoside concentrations (Li et al., 1996). Ginsenoside concentration has been shown to increase with increasing light levels from 5-30% solar radiation (Fournier et al., 2003; Park and Lee, 1993). In 1- and 2-yr old cultivated roots, light level and duration of sunflecks directly affected total ginsenoside concentration and the red: far red light ratio directly affected Rg1, Rc and Rd concentrations.

Root ginsenoside concentrations have been shown to vary significantly due to location (Assinewe et al., 2003; Li et al., 1996; Smith et al., 1996). Li and Mazza (1996) collected six 4-yr old American ginseng plants from nine commercial ginseng fields in Canada and found differences among production sites for concentrations of Rb1, Rc, Rd and total ginsenoside. They suggested that panaxadiol components (Rb1, Rc and Rd) were affected by growing conditions at these sites. Smith et al. (1996) sampled twenty 4-yr old roots from a 1-m² “homogeneous-looking” plot but found a high level of variation in total ginsenoside concentrations, similar to that reported for roots collected from variable site locations. Thus, root ginsenoside concentration in American ginseng appeared at least partly genetically controlled.

Population studies of wild American ginseng are difficult to conduct because plant sample sizes are limited by the rarity of the species and because the locations of wild populations are often unknown or carefully guarded to protect populations from poachers. In the only published study of wild American ginseng root ginsenoside

concentrations, variability of total ginsenoside concentrations in roots collected in the U.S. and Canada was found to be high, ranging from 1-16% (w/w), with the majority of roots containing 4-5% ginsenoside (Assinewe et al., 2003). Total ginsenoside concentration did not differ significantly between 4 yr old wild and cultivated roots collected in the same region in Canada (Assinewe et al., 2003). Although further studies are needed to confirm this result, these data suggest that the difference in market value between wild and cultivated roots may not be justified. Several of the wild populations showed significant variation in the levels of major ginsenosides (Assinewe et al., 2003). These authors speculated that the high level of variation observed among wild populations might be related to the high level of genetic isolation reported among wild American ginseng populations.

GENETIC MARKERS

Population genetic parameters in wild and cultivated American ginseng populations have been estimated based on two types of genetic markers: random amplified polymorphic DNA (RAPD) markers and allozyme markers (reviewed by Sunnucks, 2000). RAPD markers represent neutral sites in the genome, development is relatively inexpensive, requires little prior genetic information and markers can be amplified from DNA of low quantity or quality. However, reproducibility of polymorphisms within and among studies may be low because DNA amplification is conducted at low annealing temperatures. Allozyme markers are more reliable, but markers are less likely to represent neutral sites on the genome (markers are based on differences in protein structure, rather than differences in random, non-coding regions of DNA sequence) and marker polymorphism is generally lower than for RAPD

markers. RAPD and allozyme markers also differ in their ability to detect heterozygous genotypes (presence of different alleles at a given locus). Allozyme markers are co-dominant and can detect the presence of more than one allele for a given locus; however, RAPD markers are dominant and cannot be used to distinguish whether an individual is heterozygous at a given locus (pq) or contains two copies of the dominant allele. The dominant RAPD allele frequency (p^2) may be estimated using the frequency of the recessive RAPD allele (q^2) if Hardy-Weinberg Equilibrium ($p^2 + 2pq + q^2 = 1$) is assumed. However, high inbreeding coefficients (f) have been reported for wild American ginseng populations (Cruse-Sanders and Hamrick, 2004; Grubbs and Case, 2004) indicating that American ginseng populations are not in Hardy-Weinberg Equilibrium.

Methods that treat RAPD data as phenotypic rather than genetic and do not require the estimation of allele frequencies may be used to estimate diversity and population structure without the assumption of Hardy-Weinberg Equilibrium. Most common in the ginseng literature is the calculation of mean genetic distances from RAPD haplotypes (Bai et al., 1997; Boehm et al., 1999; Schluter and Punja, 2002) and the estimation and partitioning of Shannon's Diversity Index from RAPD marker frequencies (Schluter and Punja, 2002) within and among populations. More recently a Bayesian approach has been developed for estimating population genetic parameters from dominant marker data that incorporates uncertainty about the level of inbreeding within a population (Holsinger, 1999; Holsinger et al., 2002). If the assumption is made that the two alleles scored at each RAPD locus, dominant (p) and recessive (q) provide an adequate representation of the allele frequency distribution for a

population at the given locus (although other alleles may exist), then the Bayesian approach may be used to estimate traditional population genetic parameters (i.e. Nei's H_e and G_{st}) (Holsinger, 1999; Holsinger et al., 2002).

GENETIC VARIATION

The earliest study of genetic parameters in American ginseng calculated mean genetic distance (an estimate of genetic diversity) for a cultivated field population in Ontario using RAPD markers (Bai et al., 1997). They concluded that cultivated American ginseng was diverse and that this cultivated population was likely formed by the mixing of genetically different seed lots. When taller plants were selected, they found that genetic diversity within that group was lower than in the total population, suggesting that, “genetic factors at least partially contribute to morphological variation” (Bai et al., 1997). This study provided the first evidence that selection within American ginseng populations for superior lines based on agronomic characters was feasible. In a study of three cultivated and three wild populations of American ginseng and a Korean ginseng out-group, it was found that genetic distance was greater among than within species and that both wild and cultivated populations of American ginseng were diverse (Boehm et al., 1999). It was proposed that cultivated populations consisted largely of unimproved landraces (Boehm et al., 1999). Due most likely to drift and differential selection, Wisconsin wild and cultivated populations were found to be genetically distinct although seed for cultivated plots had been collected from wild populations in Wisconsin. Wild plants in Tennessee were distinct from all other groups, but wild plants in Pennsylvania were closely related to Wisconsin cultivated plants, suggesting that

commercial seed, purchased from farms in Wisconsin may have been introduced into wild populations in Pennsylvania.

Schluter and Punja (2002) estimated genetic diversity and population structure for four cultivated (located in Wisconsin, Nova Scotia, British Columbia and Ontario) and three wild populations (located in Quebec) of American ginseng, based on RAPD markers. Like Boehm et al. (1999), Schluter and Punja (2002) found that genetic diversity was high in both wild and cultivated populations. Cultivated and wild populations were genetically distinct from each other. Wild populations were also distinct from one another but to a lesser degree. Progeny from single mother plants exhibited much lower diversity than was estimated for randomly chosen plants from the same field plot, suggesting that much of the diversity observed in cultivated field plots resulted from the mixing of diverse lineages. Segregation of some markers among the progeny of plants that were bagged (excluding cross-pollination) indicated that parental plants were not completely inbred. Population differentiation was estimated by partitioning Shannon's Diversity Index within and among populations (Bussell, 1999). Population differentiation in wild populations was 28%, which was most similar to the level of population structure exhibited by species with mixed-mating systems (Hamrick and Godt, 1989; Hamrick and Godt, 1996; Nybom and Bartish, 2000). Although Hamrick and Godt's meta-analysis was based on estimates from allozyme markers, similar estimates of G_{st} from RAPD markers have been reported for mating system as well as taxonomic status, life form, seed dispersal mechanism and successional status (Nybom and Bartish, 2000).

In 2004, two studies were published that estimated population diversity and genetic structure in American ginseng populations based on allozyme markers (Cruse-Sanders and Hamrick, 2004; Grubbs and Case, 2004). As in earlier studies, wild populations were found to be distinct from cultivated populations and cultivated populations were observed to be diverse and experiencing high levels of gene flow. Grubbs and Case (2004) found that species-level genetic variation in wild populations sampled throughout the natural range of American ginseng was lower than reported for mixed-mating species (Hamrick and Godt, 1989) (H_e [at polymorphic loci] = 0.11 vs. 0.22), that genetic structure was higher than had been estimated by Schluter and Punja (2002) (G_{st} = 0.63 vs. 0.28) and was most similar to the estimate calculated for self-pollinating species (Hamrick and Godt, 1989) (G_{st} = 0.51). Cruse-Sanders and Hamrick (2004) found that genetic diversity in wild American ginseng sampled throughout the Appalachian mountains was similar to the level expected for mixed-mating perennial species (H_e [pooled] = 0.16 vs. 0.17) (Hamrick and Godt, 1989), but that population structure was again high (G_{st} = 0.49). Both studies reported that inbreeding (f) was high in wild populations (f = 0.62, 0.42) and Grubbs and Case (2004) reported that inbreeding was also high in cultivated populations (f = 0.65). The overall genetic profiles of wild American ginseng were attributed to a predominant life-history strategy of self-pollination (Grubbs and Case, 2004) or to the effects of harvest, i.e. reduced population sizes and isolation (Cruse-Sanders and Hamrick, 2004).

IMPACTS OF HARVEST: GENETICS

Theoretically, populations are impacted more significantly by drift, the random loss of rare alleles, when populations are small and gene flow among populations is low (Ellstrand and Elam, 1993; Hartl and Clark, 1998). Cruse-Sanders and Hamrick (2004) found that American ginseng populations that were not protected from harvest were less genetically diverse than protected populations (unprotected: $H_e = 0.70$ vs. protected: $H_e = 0.76$) and that genetic structure in unprotected populations was more than double that estimated in protected populations (unprotected: $G_{st} = 0.49$ vs. protected: $G_{st} = 0.17$). According to Grubbs and Case (2004), population size was directly correlated with diversity (H_e and P) in wild American ginseng populations. In contrast, Cruse-Sanders and Hamrick (2004) found that H_e was significantly greater in small populations suggesting that factors other than population size may affect diversity. Genetic structure in protected populations in the Appalachian Mountains was similar to the level reported for wild populations in Canada (Schluter and Punja, 2002) ($G_{st} = 0.17$ vs. 0.28), which have been protected from harvest for close to two decades (Robbins, 1998). In addition, Cruse-Sanders and Hamrick (2004) observed that expected heterozygosity was significantly greater in large plants (three- and four-leaved) as compared to small plants (one- and two-leaved) perhaps because survival rates are higher in more heterozygous individuals. Therefore, the removal of older, more genetically diverse individuals from a population could lead to the disproportionate reduction of genetic diversity in that population. Size selective harvest of American ginseng has been shown to have negative demographic consequences (Charron and Gagnon, 1991; Nantel et al., 1996;

Van der Voort et al., 2003), negative evolutionary consequences (McGraw et al., 2003) and negative genetic consequences as well (Cruse-Sanders and Hamrick, 2004).

High levels of inbreeding (f) have been reported for wild American ginseng populations (Cruse-Sanders and Hamrick, 2004; Grubbs and Case, 2004). Inbreeding can result from self-pollination or outcrossing between closely related individuals. The latter mechanism often occurs in small, isolated populations such as wild American ginseng populations; however, ginseng is known to be highly self-compatible, thus the relative influence of each of these mechanisms on inbreeding levels remains unclear. Inbreeding is generally accepted to have negative fitness consequences (Charlesworth and Charlesworth, 1987) in populations that do not have a long evolutionary history of self-pollination and have not yet purged their deleterious alleles (Carr and Dudash, 1996). Populations that are genetically diverse and experiencing substantial levels of gene flow are less likely to suffer inbreeding depression (Charlesworth and Charlesworth, 1987; Fischer and Matthies, 1998; Hedrick and Kalinowski, 2000), more likely to be able to adapt to changes in the environment (Charlesworth and Charlesworth, 1987) and ultimately have a greater probability of persistence (e.g. Frankham, 1995). Thus, genetic factors as well as demographic factors, such as stochastic events (Lande, 1988), Allee effects (Hackney and McGraw, 2001) and loss of natural colonization events (Lande, 1988) increase the probability of extinction in small, isolated populations.

Supplementation or reintroduction practices, known as “artificial seeding”, may enhance the probability of persistence in small, inbred populations by increasing

population sizes and opportunities for outcrossing. Outcrossed progeny of different inbred lines are more heterozygous and thus may be more fit than either parent, an effect referred to as “hybrid vigor”. However, when non-native seed sources are introduced into wild populations, the intraspecific hybridization of genetically divergent individuals in these populations may have negative fitness consequences, especially when introduced plants are not locally adapted (Allendorf et al., 2001; Waser, 1993; Waser and Price, 1994). Outbreeding depression can occur because introduced plants and their progeny are maladapted to the local environment (Waser and Price, 1994) or because of the breakdown of co-adapted gene complexes in hybrid progeny (Lynch, 1991; Parker, 1992; Templeton, 1986; Waser, 1993). Local adaptation is most probable in species such as American ginseng that are highly self-compatible and experiencing little gene flow among populations (Ellstrand and Elam, 1993; Parker, 1992), consequently the artificial introduction of non-native seed into wild American ginseng populations is likely to have negative fitness consequences. Artificial introductions could lead to the loss of genetic resources that confer fitness under a variety of conditions or control the expression of important agronomic traits (i.e. superior growth, superior ginsenoside levels) and more research is needed to determine the impact on artificial introductions on survival and diversity.

PROTECTION STATUS

The demand for wild American ginseng roots and the recent evidence that harvest has detrimental demographic and genetic consequences has created concern about the sustainability of harvest and conservation of wild populations. In response to this concern, American ginseng was listed under Appendix II of the Convention on

Trade of Endangered Species (CITES) in 1975. For export to remain legal under CITES regulation, the U.S. Fish and Wildlife Service (USFWS) must find that export is not detrimental to the survival of wild ginseng. The USFWS, in collaboration with individual states, must monitor wild ginseng populations and regulate ginseng harvest and export (Robbins, 1998). Dealers must register in any state in which they purchase or sell ginseng and report their transactions to the state (Robbins, 1998). Export of wild American ginseng is currently banned in Canada, but export of roots collected from wild populations is approved in 19 states in the U.S including Maryland (Robbins, 1998). Of the 19 states in which export remains legal, 13 have no listed status and the other six states (including Maryland) list ginseng as vulnerable (NatureServe, 2004). Insufficient data concerning the status of wild American ginseng has been cited as a major obstacle for determining the sustainability of harvest (Gagnon, 1999). Current state regulations on harvest require that plants must be large enough to reproduce before collection (five years), that collection each year occur after seeds mature and that seeds from harvested individuals be planted within the same population. There is concern that some states (including Maryland) allow harvest in August before fruit is fully mature and about the planting of non-local seed in harvested sites; however, illegal poaching remains the greatest obstacle to sustainability (Gagnon, 1999).

CONSERVATION AND RESOURCE MANAGEMENT

Given the high market value of wild American ginseng and the prevalence of illegal poaching, further legal limits to harvest may not be sufficient to protect wild populations. In addition, ginseng is one of the most valuable non-timber forest

products available and as such, the legal harvest of ginseng provides a strong incentive to protect forestlands. If monitored and managed properly, American ginseng harvest may provide a profitable alternative to timber harvest. To increase population sizes in the wild and reduce the impact of harvest, some managers have adopted artificial seeding practices. But it is possible that supplementation with non-native plants (often originating from cultivated field plots) will negatively affect wild populations that are locally adapted. Thus, the benefits of wild-simulated cultivation may be even greater than managed wild harvest because significant revenue can be accrued and forest integrity (and associated ecosystem services) protected without disturbing wild populations (Hill and Buck, 2000). Unless viable alternatives to unmonitored, unregulated wild harvest are found, poaching rates will probably increase. Alternatives to harvest, such as wild-simulated cultivation and artificial seeding, could be improved if more data were available comparing the genetic and phytochemical diversity of American ginseng grown in the wild and in cultivated field plots. In Maryland, harvest is legal, but the number and size of wild populations as well as the genetic and chemical diversity harbored in them is largely unknown.

In addition to the management of wild populations, knowledge of genetic and phytochemical variation in Maryland ginseng populations could enable selection of improved cultivars based on chemical superiority and help guide the strategic development of a native seed bank. Both of these outcomes could increase American ginseng revenue and minimize the impacts of wild harvest in Maryland. Wild populations may contain genes or gene complexes that provide disease resistance, confer fitness under a variety of conditions or control the expression of superior

agronomic characters not found in cultivated populations. Maintenance of high levels of genetic variation within and among populations and the protection of genetic resources associated with characters of agronomic importance would make selection for these characters feasible in the future.

Genetic resources can be conserved *in situ*, in wild populations, or *ex situ*, in seed bank collections. Ideally, wild populations should be protected in their native environment, which is why the USFWS is required to restrict harvest and ensure species persistence. However, *ex situ* methods provide a secondary measure to conserve genetic resources and to make germplasm more available for cultivation, supplementation and reintroduction efforts (Marshall, 1989; Maunder et al., 2004). A disadvantage of *ex situ* conservation is that seed banks are labor intensive and expensive to develop and maintain. As opposed to a collection of seed from all wild Maryland plants, a core collection would be composed of plants that are genetically representative of the species, but not redundant. Brown (1989) suggests that a core collection should contain 75% of the total diversity of the whole collection, but be small enough to manage easily. Selection of a core should be based on the geographic origin, genetic characteristics and valuable traits, such as chemical concentration, of each accession. Thus, data concerning the level, type and distribution of genetic and phytochemical diversity in wild and cultivated populations of American ginseng are necessary to guide the development of an American ginseng seed bank. Seed banks developed in the absence of these data are more likely to be genetically redundant and fail to represent the variability contained in wild and cultivated populations.

Chapter 2: Genetic Diversity

ABSTRACT

American ginseng (*Panax quinquefolius*) is a highly valued medicinal herb that is becoming increasingly rare due to habitat destruction and over-harvesting of wild populations. In an effort to support declining populations, supplementation or reintroduction of wild populations, known as “artificial seeding” has been widely adopted. However, supplementation efforts are controversial because introduction of non-native plants into cultivated and wild populations threatens to pollute native germplasm. In this study, Random Amplified Polymorphic DNA (RAPD) markers were used to estimate genetic diversity and genetic structure of cultivated and wild populations from the Appalachian and Piedmont regions of Maryland. My results corroborated earlier findings that American ginseng populations are diverse at the species level. However, wild populations exhibited a large range of within-population diversities with a substantial amount of genetic variation partitioned among populations. Wild Maryland germplasm was distinct from exotic commercial germplasm but plants cultivated from Maryland seedstock grouped predominantly with wild populations suggesting a local origin of these crops. Plants from one putatively native population clustered with exotic commercial plants indicating that this population was derived from non-native sources. Thus, wild American ginseng populations in Maryland appear to be influenced by high levels of drift and isolation as well as genetic contamination via artificial seeding.

INTRODUCTION

American ginseng (*Panax quinquefolius*) is a perennial herb that grows in the understory of mature deciduous forests throughout the eastern U.S. and Canada and is widespread along the Appalachian mountain range where it is native in Maryland. Ginseng roots are highly valued for their medicinal properties in Eastern traditions and wild American ginseng has been harvested for export to Asia since its discovery over 300 years ago (Persons, 1994). American ginseng has been cultivated in North America since the late 1800s, but cultivated roots are considered to be less medicinally potent and substantially less valuable than wild roots (Persons, 1994). The high commercial value of wild roots has led to over-harvest, which has contributed to the decline and extinction of American ginseng populations throughout its native range (Charron and Gagnon, 1991; Cruse-Sanders and Hamrick, 2004; Nantel et al., 1996). American ginseng has been federally regulated under Appendix II of the Convention for International Trade of Endangered Species (CITES) since 1975. While ginseng export is illegal in Canada, export remains legal but restricted in 19 states in the U.S., including Maryland (Robbins, 2000). Restrictions for harvesting wild ginseng include age and harvest season limits and certification of roots prior to export (Robbins, 2000). Due to lack of funding to adequately monitor and enforce these programs, the impacts of legally harvesting and illegally poaching American ginseng remains largely unknown (Robbins, 2000). The lack of information concerning the population and conservation status of this species has been cited as a major obstacle for determining the sustainability of harvest (Gagnon, 1999; Robbins, 2000). In Maryland, American ginseng is listed as rare or uncommon

(Maryland Wildlife and Heritage Division, 2001), although only limited biological census data is available on the number or integrity of wild populations.

The estimation of genetic parameters can provide useful information concerning the status and integrity of wild populations. In Maryland, wild ginseng populations are generally small and isolated, but little is known about the genetic status of these populations. In other parts of its range, varying levels of within and among population diversity have been reported for wild American ginseng populations. Earlier studies of American ginseng populations, based on RAPD markers, concluded that diversity was high (Boehm et al., 1999; Schluter and Punja, 2002) and genetic structure was suggestive of a mixed-mating system (Schluter and Punja, 2002); however, only a small number of protected Canadian populations were considered. More recently, studies based on allozyme markers (Cruse-Sanders and Hamrick, 2004; Grubbs and Case, 2004) found that genetic structure among many wild populations was much higher than reported by Schluter and Punja (2002), and that genetic structure (G_{st}) and inbreeding (f) were higher and within-population diversity lower (H_e) than expected for a mixed-mating species (Cruse-Sanders and Hamrick, 2004; Grubbs and Case, 2004). However, wild populations that were protected from harvest exhibited a genetic profile more similar to that reported by Schluter and Punja (2002). These results suggest that a mixed-mating strategy may account for the level and partitioning of diversity reported in protected populations, but that harvest may in large part be responsible for the high levels of drift and inbreeding observed in unprotected populations (Cruse-Sanders and Hamrick, 2004). High levels of inbreeding and drift in harvested populations may lead to declines in

fitness (Charlesworth and Charlesworth, 1987; Keller and Waller, 2002), failure to adapt to environmental changes and disease and ultimately extinction (e.g. Ellstrand and Elam, 1993; Frankham, 1995). The results of these studies raise serious concerns about the sustainability of American ginseng harvest.

In an effort to supplement wild populations and reduce the effects of harvest, seed from cultivated field populations may be introduced into wild populations (Anderson et al., 2002); however, this practice, known as “artificial seeding”, is controversial because the origin of seed is rarely known and may not be local. Cultivated field populations have been reported to be diverse (Bai et al., 1997; Boehm et al., 1999; Grubbs and Case, 2004; Schluter and Punja, 2002) and genetically distinct from wild populations (Boehm et al., 1999; Grubbs and Case, 2004; Schluter and Punja, 2002). There are no known cultivars of American ginseng and it has been suggested that field plots consist of unimproved landraces (Boehm et al., 1999). The artificial introduction of seed from these cultivated field plots into wild populations may increase genetic diversity and reduce inbreeding in the supplemented population. However, mal-adaptation to the local environment (Waser and Price, 1994) and the breakdown of co-adapted gene complexes (Lynch, 1991; Templeton, 1986) in hybrid progeny may lead to a loss of fitness, or outbreeding depression. Outbreeding depression is most probable in species such as American ginseng that appear to experience low levels of gene flow and high rates of self-pollination (Allendorf et al., 2001; Ellstrand and Elam, 1993; Parker, 1992). Consequently, it is plausible that the artificial introduction of non-native seed into wild American ginseng populations could have negative fitness consequences. Artificial introductions could lead to the

loss of genetic resources that confer fitness under a variety of conditions or control the expression of important agronomic traits (i.e. superior growth, superior ginsenoside levels) and more research is needed to determine the impact on artificial introductions on these traits as well as genetic diversity and survival.

Given the demand for wild American ginseng, further restrictions to harvest may not be sufficient to protect wild populations. In addition, wild American ginseng harvest supplies significant revenue to rural communities and provides an incentive to protect forests. Population genetic data provide information that may be used to improve the management of wild and cultivated populations for long-term sustainability. The goals of this study were to use random amplified polymorphic DNA (RAPD) markers to assess the current status of genetic diversity in wild and cultivated populations in Maryland for future planning and management of wild populations and to guide the strategic development of a Maryland seed bank. Seed banks developed in the absence of genetic data may be redundant or fail to represent the genetic variability contained in wild and cultivated populations. Although *in situ* conservation of local genetic variation is preferred, *ex situ* conservation of genetic resources in a well-developed native Maryland seed bank would provide a method to protect native germplasm and make available native seed source for reintroductions, supplementation and cultivation.

MATERIALS AND METHODS

Sample Collection

Mr. Robert Trumbule, Plant Protection Specialist at the Maryland Department of Agriculture, collected leaf samples from wild and cultivated populations in four

counties in Maryland (Alleghany, Garrett, Fredrick and Washington) during the summers of 2002 and 2003 (Table 1). Leaves from cultivated populations were obtained from commercial ginseng growers in Maryland. In 2002, 43 individuals were sampled from 14 wild populations in Maryland. Populations were spatially distinct and potential subpopulations were categorized as separate populations to reduce the likelihood of sampling across distinct populations. In 2003, leaves were collected from 10-23 individuals from each of the largest populations surveyed in 2002 (Table 1). Wild leaf samples collected in 2002 could not be pooled with those collected in 2003 because plants were not tagged and redundant sampling could not be ruled out (tagging was considered a threat to plant persistence given the harvest pressure in Maryland).

Cultivated plants were collected from nine field populations and samples were pooled over the two years. Cultivated samples were categorized as native (plants allegedly grown from native Maryland seed stocks) or exotic (plants grown from exotic commercial seed from Tennessee or Wisconsin) (Table 1). Cultivated populations were grouped rather than considered individually because sample sizes were generally small (<10) and to distinguish between plants cultivated from exotic seed and those cultivated from native seed (for the list of cultivated populations see Appendix A). Root samples that were collected for a related study were used when no leaf material was available from a plant. Leaf and root samples were freeze-dried and kept at -20°C until DNA extraction.

Table 1. Population category, geographic location, number of populations, sample size and estimated population size of wild and cultivated American ginseng populations in Maryland considered in this study (leaf samples were used in this study unless noted otherwise).

Population Category	Geographic Location^a	Populations Sampled	Sample Size^c	Population Size^d
Wild 2002	A, G, W	14	43	n/a
Cultivated 2002-3:				
Native seed	A, F, G, W	9	38 (12)	n/a
Exotic seed	A, W (TN, WI) ^b	3	11 (6)	n/a
Wild 2003:				
	G, W	7	130	n/a
P1	G	1	21	27
P2	G	1	16	16
P3	G	1	20	27
P4	G	1	21	30
P5	W	1	23	60
P6	W	1	10	20
P7	W	1	19	30
Total			240	

^a A = Allegheny Co., F = Fredrick Co., G = Garrett Co., W = Washington Co.,

^b Commercial seed purchased from farms in TN = Tennessee and WI = Wisconsin.

^c Root sample sizes are provided in parentheses.

^d Approximate population sizes.

DNA Extraction

Leaves were freeze-dried for 24 hrs and immediately ground to a fine powder in a 1.5-ml microcentrifuge tube with a micropestle. Roots were freeze-dried for 72 hrs and ground in a Wiley Mill (20 mesh) (Foss Tecator AB, Höganäs). DNA was isolated from approximately 20 mg of leaf or root powder following the standard DNeasy® Plant Mini Kit protocol (Qiagen, Valencia). DNA was eluted with 150 µl

DNeasy AE buffer twice and the DNA concentration was estimated spectrophotometrically (A_{260}). DNA concentrations did not differ greatly and an aliquot of all samples was diluted 1:4 (5-10 ng μl^{-1}) for use in RAPD PCR. Samples that did not amplify well were diluted until amplification improved.

RAPD PCR

A total of 180 decamer primers were initially screened for polymorphisms on a subset of eight leaf samples collected from wild and cultivated populations. Primers were obtained either from Qiagen (Valencia) (based on the sequences provided by Operon Technologies, Alameda) or from the Nucleic Acid Protein Service Unit at the University of British Columbia (Vancouver). Twenty decamers that produced highly reproducible polymorphic markers were selected for RAPD analysis of all 240 samples (Table 2 and Figure 4). Polymorphic markers were scored twice for twenty representative samples and percent error (number of mismatches/total number of marker comparisons x 100) was calculated to estimate marker reproducibility in this study. Less than 5% scoring error was calculated for the twenty replicated samples, indicating that at least 95% of the RAPD markers scored were reproducible. Polymorphic markers were named according to decamer code and the size of the amplified fragment (Table 2).

Table 2. List of RAPD decamer codes and sequences, number of polymorphic and monomorphic markers amplified by each decamer and sizes of polymorphic markers.

Decamer Code	5' to 3' Sequence	Monomorphic Markers	Polymorphic Markers	Polymorphic Marker Size (bp)
OA07	GAAACGGGTG	2	3	485, 950, 1000
OA12	TCGGCGATAG	1	2	485, 515
OD05	TGAGCGGACA	2	1	525
OE01	CCCAAGGTCC	1	1	700
OE09	CTTCACCCGA	1	1	650
OE16	GGTGA CTGTG	1	3	310, 590, 950
OF02	GAGGATCCCT	1	1	600
OF20	GGTCTAGAGG	1	2	750, 925
OG11	TGCCCGTCGT	1	2	600, 1100
OG13	CTCTCCGCCA	0	1	900
OG16	AGCGTCCTCC	1	1	900
OU01	ACGGACGTCA	1	3	375, 600, 925
OU02	CTGAGGTCTC	2	1	500
OZ03	CAGCACCGCA	0	3	775, 1000, 1600
OZ04	AGGCTGTGCT	2	4	575, 700, 750, 1800
OZ17	CCTTCCCACT	0	1	1900
UBC221	CCCGTCAATA	0	2	1000, 1400
UBC223	GATCCATTGC	2	4	625, 975, 1400, 1700
UBC226	GGGCCTCTAT	2	1	700
UBC227	CTAGAGGTCC	1	1	1300
Total		22	38	

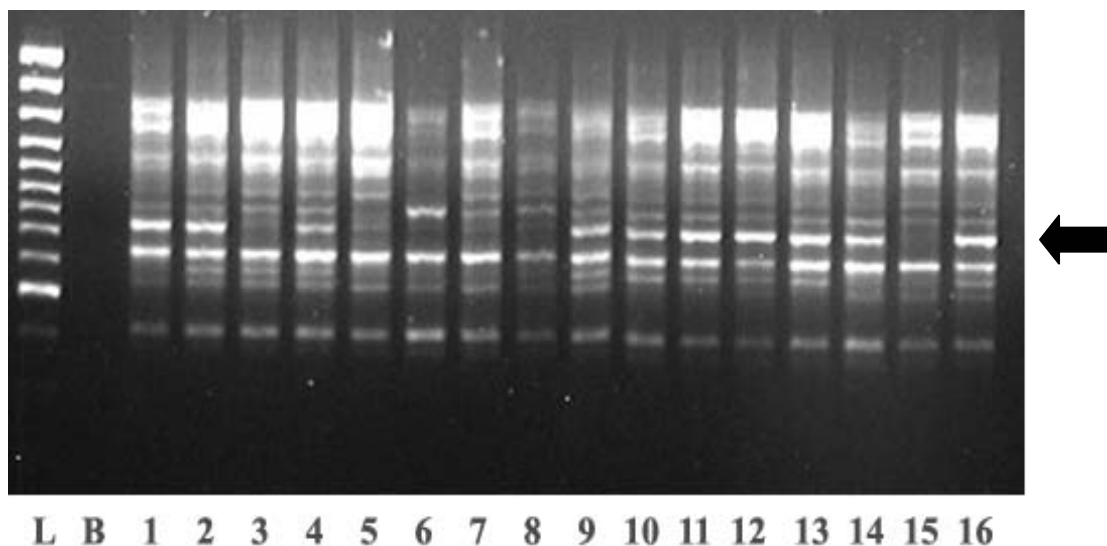


Figure 4. PCR amplification of RAPD marker OG16-700 with DNA isolated from American ginseng leaf and root tissue. L: 1KB ladder, B: negative control, 1-4: plants cultivated from native seed, 5-8: plants cultivated from exotic seed, 9-16: wild plants. Arrow indicates polymorphic marker.

Mixtures for RAPD PCR (25 μ L) reactions contained 20 ng DNA, 10 mM KCL, 10 mM (NH₄)SO₄, 20 mM Tris-HCL, 3 mM MgSO₄, 0.1% Triton X-100, 0.1 mM dNTPs, 35 ng of a single decamer and 1 unit taq polymerase (NEB, Beverly). Amplification was performed on a GeneAmp® PCR system (Perkin-Elmer, Boston) or a Eppendorf Mastercycler Gradient (Brinkmann Instruments, Inc., Westbury) DNA thermocycler for 45 cycles according to the procedure of Williams et al. (1990). Approximately 15 μ L of the reaction was loaded onto a 2 % agarose gel containing ethidium bromide (0.5 μ g ml⁻¹) and PCR fragments were separated by electrophoresis (2 hrs, 120 V). RAPD fragments were illuminated under UV light and images were captured with an Eagle Eye II instrument using EagleSight software (Stratagene, La Jolla).

Genetic Diversity

Polymorphic markers were scored as present (1) or absent (0) for each individual and were assumed to represent neutral, independent genetic loci. Samples that were missing data for greater than 10% of loci were excluded from analyses. The percent of markers that were polymorphic (P) within groups or populations provided an estimate of diversity (marker frequencies <1% considered monomorphic: 99% criterion). The multilocus RAPD phenotype was treated as a haplotype and a dissimilarity index was calculated based on marker sharing among individuals. Genetic distance, or dissimilarity, was calculated for all pair-wise combinations of individuals as the complement to the simple-matching (Sneath and Sokal, 1973), the Nei-Li (or Dice) (Dice, 1945; Nei and Li, 1979) and Jaccard's (Gower, 1971; Jaccard, 1908) similarity coefficients. Mean pair-wise genetic distance within and among populations provided estimates of diversity.

Three estimates of genetic distance were considered in this study and Mantel tests were used to assess the correlation between distance matrices of each of these indices using NTSYSpc 2.0 (Rohlf, 1998). Significance of the Mantel tests was assessed using permutation tests with 1000 permutations. The dissimilarity indices used in this study differed in their inclusion and weighting of shared absence (0,0) matches and shared presence (1,1) matches. Although the simple matching coefficient is commonly used to analyze RAPD marker data, some researchers suggest that a 0,0 match is irrelevant because the null (band absence) response of a dominant marker can be derived from a variety of genetic anomalies and may not represent a shared character between the samples (Brown-Guedira et al., 2000).

However, the exclusion of these potential null similarities in the Jaccard coefficient biases estimates towards greater diversity (or distance) among samples. Although the inclusion of monomorphic markers would minimize bias towards greater diversity, the inclusion of monomorphic markers in the analyses did not change the conclusions and made comparisons between this study and those in the literature more difficult. Thus, analyses were conducted solely on polymorphic markers.

Expected heterozygosity ($H_e = 1 - \sum p_i^2$; where i is the number of alleles) (Nei, 1973) is a measure of within-population diversity calculated from allele frequency data. However, because RAPD markers are dominant and heterozygous genotypes cannot be distinguished from homozygous dominant genotypes, population allele frequencies can only be estimated from RAPD data if Hardy-Weinberg equilibrium is assumed. However, significant deviations from Hardy-Weinberg equilibrium (high inbreeding levels) have been reported for wild American ginseng populations (Cruse-Sanders and Hamrick, 2004; Grubbs and Case, 2004). Recently, a Bayesian approach, that incorporates uncertainty about inbreeding levels within sampled populations, has been developed to estimate genetic parameters from dominant markers (Holsinger, 1999; Holsinger et al., 2002). Analogs of Nei's (1973) expected heterozygosity ($H_{e\beta}$), mean expected heterozygosity (mean $H_{e\beta}$ is equivalent to $H_{s\beta}$ if only polymorphic markers are included in the analyses) and total heterozygosity ($H_{t\beta}$) were estimated using Hickory V0.1 software (Holsinger and Lewis, 2003) (see section on *Genetic Structure* for more details about this method). Expected heterozygosity ($H_{e\beta}$) provided an estimate of within-population diversity, mean $H_{e\beta}$

($H_{s\beta}$) provided an estimate of average population diversity and total heterozygosity ($H_{t\beta}$) provided an estimate of species-level diversity (all individuals pooled).

Genetic Relationships

Principal component analysis and multidimensional scaling techniques were used to visualize the relationships among individuals or populations in three or fewer dimensions. The variance-covariance matrix of RAPD marker frequencies for each population was reduced to three components using principal component analysis (PCA) in SAS (PROC PRINCOMP) (SAS Institute Inc., 2002). The variance explained by each principle component provided a measure of the fit of the model. The distribution of 197 individual plants, based on the pair-wise simple-matching genetic distance among them, was reduced to two dimensions using metric multidimensional scaling (MDS) in SAS (PROC MDS) (SAS Institute Inc., 2002). All samples collected from the seven wild populations in 2003 and all cultivated samples were included in this analysis. The correlation coefficient of the original distance data by the distance data output from the MDS model was calculated using SAS (PROC CORR) (SAS Institute Inc., 2002) to estimate the fit of this model.

Genetic Structure

Analogs of F_{st} (Wright, 1951) were estimated for seven wild American ginseng populations sampled in Maryland in 2003 using a Bayesian approach developed for dominant markers (Holsinger et al., 2002) and by partitioning Shannon's Diversity Index (based on RAPD marker frequencies) among populations (Bussell, 1999). These two estimation methods do not assume that sampled

populations are in Hardy-Weinberg equilibrium. The Bayesian approach was used to estimate analogs of Weir and Cockerham's θ (1984) (θ_β) and Nei's G_{st} (1973) ($G_{st\beta}$) as implemented in the program Hickory V1.0 (Holsinger and Lewis, 2003) with the default parameters as described by Holsinger et al. (2002). Estimates of θ_β and $G_{st\beta}$ provided measures of genetic differentiation (or structure) among the sampled populations. All Bayesian models (full, $f = 0$, $\theta = 0$ and f free) were analyzed and a best-fit model was chosen based on the minimum Deviance Information Criterion (DIC) (Spiegelhalter et al., 2002) and the entropy statistic (Ie) (Holsinger and Wallace, 2004). The mean of the posterior distribution of θ_β was reported for each marker separately and globally for all markers. Bayesian analogs of Nei's (1973) expected heterozygosity ($H_{e\beta}$), mean expected heterozygosity ($H_{s\beta}$) and total heterozygosity ($H_{t\beta}$) were estimated for all wild populations and $G_{st\beta} = 1 - (H_{s\beta}/H_{t\beta})$ based on the mean of the posterior distribution for each of the dominant allele frequencies (Holsinger, 1999). Confidence intervals based on the posterior distributions of Bayesian estimates provided a measure of estimate reliability (Holsinger, 1999).

For comparison, Shannon's Diversity Index was used to partition RAPD diversity within and among populations as described by Bussell (1999). Partitioning of Shannon's diversity required no assumption of Hardy-Weinberg equilibrium because data were treated as phenotypic rather than genetic. Markers that were monomorphic within a population but polymorphic within the species were included and Shannon's Diversity Index for each marker was calculated for each population as $H'_j = -\sum p_i \log p_i$, where p_i was the relative frequency of the presence or absence of a

RAPD marker in that population (Bussell, 1999). The average diversity across populations was calculated as $H'_{pop} = 1/n \sum H'_j$ (where n is the number of populations) and the diversity of the species was calculated as $H'_{sp} = -\sum p_i \log p_i$, where p_i was the frequency of the presence or the absence of a RAPD band in the whole sample (all 130 wild samples). The component of phenotypic variation among populations was calculated as $G_{st} = 1 - (H'_{pop} / H'_{sp})$. Overall estimates of diversity were calculated from the average per-marker values of H'_j , H'_{pop} , H'_{sp} and G_{st} .

Population Integrity

All pair-wise combinations of wild populations and production types (exotic or native) were tested for significant differences in marker frequencies. In the program Tools for Population Genetic Analysis (Miller, 1998), a Markov Chain Monte Carlo approximation of Fisher's exact test of marker frequencies was employed as described by (Raymond and Rousset, 1995). The χ^2 values and probabilities were reported from the Combined Fisher's Probability Test of overall significance across all RAPD markers. The magnitude of the combined Fisher's χ^2 value provided an indication of the level of differentiation among populations or production types.

RESULTS

Genetic Diversity

Of the 180 decamers screened for polymorphism, twenty (11%) amplified thirty-eight highly reproducible polymorphic markers (66% of the total number of markers amplified) (Table 2). Only markers that demonstrated clear, high intensity

signals were selected for further analyses (Figure 4). Each decamer amplified from 1 to 4 polymorphic markers that ranged in size from 300 to 2000 bp (Table 2).

Table 3 presents estimates of total diversity for wild and cultivated American ginseng grown in Maryland (production-type) and estimates of within-population diversity in wild populations. All three genetic distance matrices were highly correlated ($r > 0.95$; $P = 0.002$ for 1000 permutations), thus any single distance metric was sufficient to describe the relative relationships among individuals and populations. For clarity, further analyses were based on simple-matching genetic distance estimates; however, all genetic distance estimates were included in Table 3 to facilitate comparisons with studies that employed different genetic distance metrics.

Total genetic diversity was high in exotic cultivated ($H_{t\beta} = 0.38$, $SM = 0.37$), native cultivated ($H_{t\beta} = 0.33$, $SM = 0.27$) and wild ($H_{t\beta} = 0.33$, $SM = 0.33$) American ginseng populations (Table 3) (see Appendix A for SM estimates of individual cultivated populations). However, diversity within individual wild populations P1-P7 ranged from almost complete identity (P4: $H_{t\beta} = 0.05$, $SM = 0.03$) to high levels of diversity (P7: $H_{t\beta} = 0.38$, $SM = 0.42$). In population P4, 18 out of the 38 markers were absent in all individuals and two out of the remaining 20 markers were polymorphic (5%). In comparison, all 38 markers were polymorphic in population P7 ($P = 100$). Mean within-population diversity was lower in wild populations in Garrett County ($H_{t\beta} = 0.11$, $SM = 0.09$) as compared to populations in Washington County ($H_{t\beta} = 0.33$, $SM = 0.31$).

Table 3. Estimates of total genetic diversity: mean genetic distance, total heterozygosity ($H_{t\beta}$) and percent polymorphic markers (P), for wild and cultivated American ginseng production types collected in Maryland in 2002-3. Estimates of within-population diversity: mean genetic distance, expected heterozygosity ($H_{e\beta}$) and percent polymorphic markers (P), for wild American ginseng populations sampled in Maryland in 2003. N = sample size. Standard deviations are in parentheses.

Production Type	N	Mean NL^a	Mean SM^b	Mean JD^c	$H_{t\beta}$	P^d
Wild 2002	43	0.34 (0.16)	0.34 (0.17)	0.48 (0.18)	0.31 (0.01)	97
Cultivated 2002-3:	67	0.32 (0.15)	0.37 (0.16)	0.47 (0.17)	0.38 (0.01)	90
Native seed	50	0.23 (0.11)	0.27 (0.11)	0.37 (0.14)	0.33 (0.01)	90
Exotic seed	17	0.31 (0.09)	0.37 (0.11)	0.47 (0.11)	0.38 (0.01)	90
Wild 2003	130	0.34 (0.14)	0.33 (0.15)	0.49 (0.16)	0.33 (0.01)	100
Wild 2003	N	Mean NL^a	Mean SM^b	Mean JD^c	$H_{e\beta}$	P^d
P1	21	0.05 (0.06)	0.06 (0.06)	0.09 (0.10)	0.08 (0.01)	32
P2	16	0.11 (0.07)	0.10 (0.07)	0.19 (0.12)	0.12 (0.01)	29
P3	20	0.21 (0.10)	0.21 (0.09)	0.33 (0.14)	0.19 (0.01)	55
P4	21	0.02 (0.02)	0.03 (0.04)	0.03 (0.03)	0.05 (0.01)	5
P1-P4 ^e	78	0.10	0.09	0.16	0.11	63
P5	23	0.31 (0.12)	0.32 (0.11)	0.46 (0.14)	0.30 (0.01)	82
P6	10	0.25 (0.09)	0.26 (0.08)	0.39 (0.12)	0.24 (0.01)	61
P7	17	0.40 (0.19)	0.42 (0.17)	0.55 (0.19)	0.38 (0.01)	100
P5-P7 ^f	52	0.32	0.33	0.35	0.31	100

^a [NL] Nei and Li's genetic distance.

^b [SM] simple-matching genetic distance.

^c [JD] Jaccard's genetic distance.

^d 99% polymorphism criterion.

^e Garrett County.

^f Washington County.

In Table 4, estimates of mean genetic distance among wild populations and cultivated production types are presented. Mean simple-matching genetic distance was high among all populations and ranged from 0.20 (P2 and P4) to 0.66 (P2 and plants grown from exotic seed) (Table 4). Populations from Washington County (P5-

P7) exhibited similar levels of diversity within and among populations; however, populations in Garrett County (P1-P4) exhibited much higher levels of diversity among populations (Table 3 and 4). Plants cultivated from Maryland seed stock were most genetically similar to populations P1 and P4 (Table 4). Exotic commercial plants were dissimilar from all groups; however, wild population P7 was more similar to exotic plants (SM = 0.43) than native (SM = 0.47).

Table 4. Mean pair-wise simple-matching genetic distance among wild populations (P1-P7) and cultivated production types (exotic and native) of American ginseng grown in Maryland.

		Wild							Cultivated	
		P1	P2	P3	P4	P5	P6	P7	Exotic	Native
Wild:	P1									
	P2	0.35								
	P3	0.32	0.22							
	P4	0.28	0.20	0.26						
	P5	0.31	0.39	0.37	0.38					
	P6	0.29	0.30	0.31	0.33	0.31				
	P7	0.41	0.52	0.49	0.51	0.44	0.46			
Cultivated:	Exotic	0.51	0.66	0.60	0.61	0.49	0.54	0.43		
	Native	0.24	0.31	0.32	0.26	0.36	0.33	0.47	0.53	

Relative frequencies of eight RAPD markers found at highest frequency in plants cultivated from exotic seed sources are reported in Table 5. Exotic plants and wild plants in population P7 exhibited high frequencies of four RAPD markers that were absent or observed at low frequency in all other populations (OA07-1000, OU01-600, UBC226-700 and UBC227-1300) (Table 5). Exotic plants, wild

populations P5 and P7 exhibited high frequencies of additional four RAPD markers that were absent or at low frequency in all other populations (OA12-485, OF20-750, OF20-925 and OZ04-1800) (Table 5). However, the number of individuals in which these markers were present was consistently lower in population P5 as compared to population P7 and exotic plants (Table 5). Markers F20-750 and Z04-1800 were present at low frequency in plants cultivated from native seed sources.

Table 5. Relative frequencies of eight RAPD markers observed in high frequency in American ginseng plants cultivated from exotic seed in Maryland.

	Wild							Cultivated	
	P1	P2	P3	P4	P5	P6	P7	Exotic	Native
OA12-485	-	-	-	-	0.26	-	0.32	0.76	-
OF20-750	-	-	-	-	0.13	-	0.50	0.71	0.10
OF20-925	-	-	-	-	0.09	-	0.61	0.65	-
OZ04-1800	-	-	-	-	0.13	-	0.74	0.82	0.12
OA07-1000	-	-	-	-	-	-	0.13	0.59	-
OU01-600	-	-	-	-	-	-	0.47	0.65	-
UBC226-700	-	-	-	-	-	-	0.16	0.35	-
UBC227-1300	-	-	-	-	-	-	0.39	0.59	-

Markers with frequencies $\leq 5\%$ (95% criterion) indicated by a dash.

Genetic Relationships

Relationships among wild populations (P1-P7) and cultivated production types (native and exotic) based on marker frequency data are displayed as plots of the first three principal components, which accounted for 83% of the total variance (Figure 5). The first principal component (PCA1) explained 49% of the variance and

from left to right clearly separated exotic plants from plants cultivated from native seed sources. Wild populations P1-P6 were more similar to native plants than exotic, but population P7 was more similar to exotic plants than native. PCA2 accounted for 19% of the variance and separated wild population P1 from the rest of the populations. PCA3 accounted for 15% of the variance and grouped wild populations P2, P3, P5 and P6 separately from native cultivated plants and wild populations P1 and P4. Although native and exotic groups were clearly distinguished, a large amount of variation appeared to exist among wild populations.

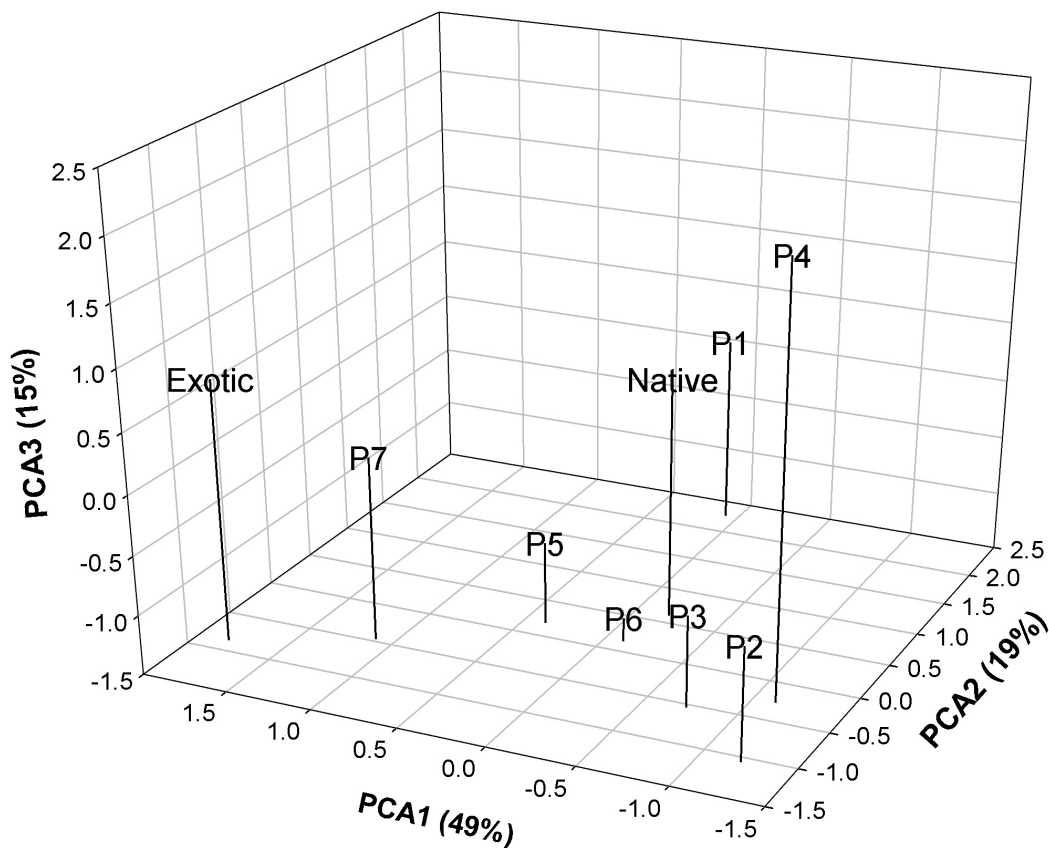


Figure 5. Principal component analysis of marker frequencies observed in wild populations (P1-P7) and cultivated production types (exotic or native) of American ginseng grown in Maryland. The percent of variance explained by each component is given in parentheses (total = 83%).

Relationships among the 197 individual samples included in the seven wild populations and the two cultivated production types (native and exotic) were displayed in two dimensions based on the simple-matching genetic distance among them (model fit $[r] = 0.91$) (Figure 6). Plants in P1-P4 (1-4) were tightly clustered and formed distinct groups that appeared to be closely related to plants cultivated from native seed sources (N). Plants cultivated from exotic seed (E) overlapped with individuals in wild population P7 and formed a group of individuals that were diverse (diffuse groupings) and clearly distinct from native plants (N, 1-4).

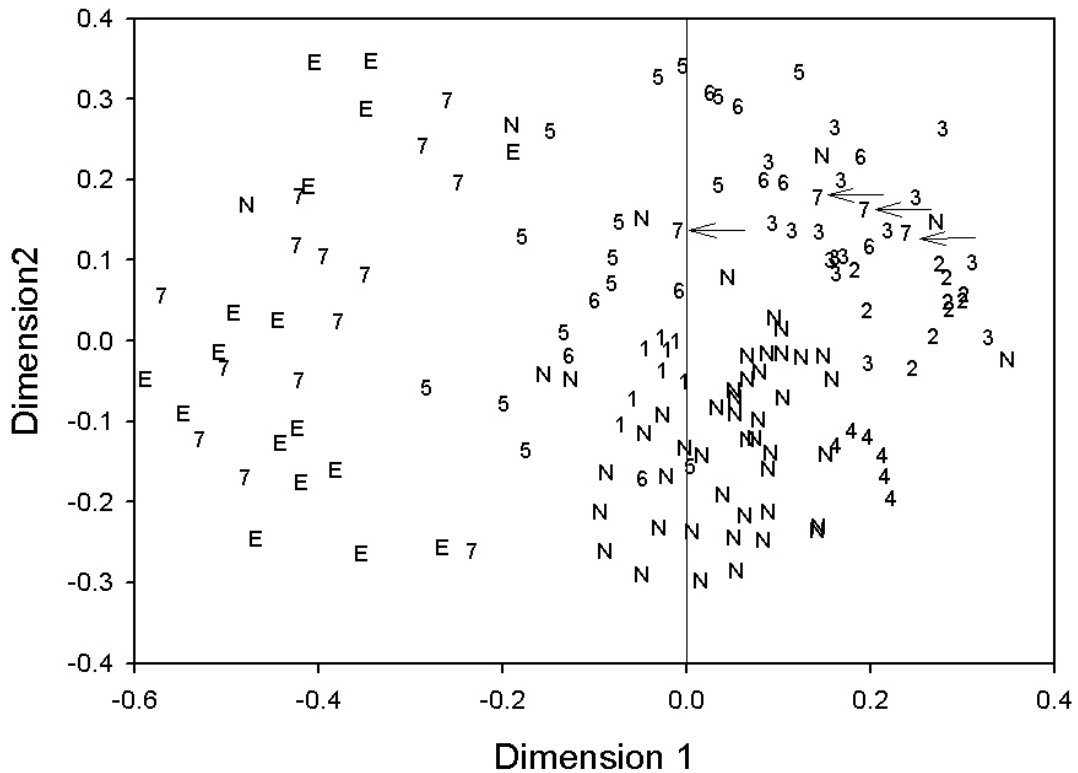


Figure 6. Multidimensional scaling plot of American ginseng samples from wild populations (1-7) and cultivated production types (native [N] and exotic [E]) ($r = 0.91$). All 197 plants included in this study are represented in this figure, but some individuals are hidden because their RAPD haplotypes were identical.

Plants in wild populations P5 and P6 (5 and 6) were located intermediate to the native and exotic groups and were diffuse, appearing to be as closely related to individuals in other populations as to those within their own population (Figure 6). Within population P7, 15 out of 19 plants were closely related to exotic plants and the other four plants were more similar to wild populations P1-P4 (indicated by arrows in Figure 6). While P7 contained two distinct groups of individuals, populations P5 and P6 were positioned intermediate to exotic and native plants and no clear grouping were observed. Two plants allegedly cultivated from native Maryland seed stocks grouped more closely with plants from population P7 and exotic commercial plants.

Genetic Structure

Of the Bayesian models that were run in Hickory V1.0 (Holsinger and Lewis, 2003), the full and the f free models provided the best fit (full model: DIC = 737, I_e = 2.01; f free model: DIC = 764, I_e = 2.04). Although estimates from each of the models were similar, the full model was used because it was considered to be more robust (Holsinger and Lewis, 2003). Estimates of genetic structure were large according to all methods used and indicated that 41-46% of the total genetic variation was partitioned among wild American ginseng populations in Maryland (Table 6). Significant genetic structure existed among populations ($P < 0.05$) and populations in Garrett County were more highly differentiated than those in Washington County (Table 6). Some individual markers exhibited much lower estimates of θ_β than the global estimate (Table 7). Eight markers present predominantly in wild populations in Washington County (P5 and P7) and exotic commercial plants (Table 5) yielded estimates of θ_β that ranged from 0.10 to 0.13 (Table 7).

Table 6. Estimates of genetic structure for wild populations of American ginseng in Maryland (P1-P7) based on the Bayesian analog of Weir and Cockerham's θ , the Bayesian analog of Nei's G_{st} and the partitioning of Shannon's Diversity Index among populations (G_{st}). Standard deviations are in parentheses.

Parameter	Symbol	Wild	P1-P4 ^a	P5-P7 ^b
Bayesian analog of θ	θ_β	0.46 (0.03)*	0.56 (0.05)*	0.16 (0.03)*
Bayesian analog of G_{st}	$G_{st\beta}$	0.41 (0.02)*	0.52 (0.03)*	0.11 (0.02)*
Shannon's G_{st}	G_{st}	0.45	0.52	0.20

^a Wild populations in Garrett County.

^b Wild populations in Washington County.

* $P < 0.05$.

Table 7. Per-marker Bayesian analogs of Weir and Cockerham's θ (θ_β) estimated for wild populations of American ginseng grown in Maryland. Standard deviations are in parentheses.

Marker	θ_β	Marker	θ_β	Marker	θ_β
OA07-875	0.51 (0.13)	OF20-925	0.13 (0.06)	OZ04-700	0.64 (0.11)
OA07-950	0.40 (0.12)	OG11-600	0.48 (0.12)	OZ04-750	0.87 (0.06)
OA07-1000	0.11 (0.06)	OG11-1100	0.42 (0.12)	OZ04-1800	0.13 (0.07)
OA12-485	0.13 (0.06)	OG13-900	0.47 (0.13)	OZ17-1900	0.35 (0.12)
OA12-515	0.85 (0.08)	OG16-700	0.65 (0.11)	UBC221-1000	0.86 (0.07)
OD05-525	0.68 (0.12)	OU01-375	0.65 (0.11)	UBC221-1400	0.82 (0.08)
OE01-700	0.35 (0.10)	OU01-600	0.10 (0.05)	UBC223-625	0.58 (0.12)
OE09-650	0.39 (0.11)	OU01-925	0.72 (0.11)	UBC223-975	0.41 (0.11)
OE16-310	0.66 (0.12)	OU02-500	0.79 (0.10)	UBC223-1400	0.53 (0.12)
OE16-590	0.37 (0.12)	OZ03-775	0.51 (0.13)	UBC223-1700	0.83 (0.08)
OE16-950	0.51 (0.12)	OZ03-1000	0.21 (0.09)	UBC226-700	0.10 (0.05)
OF02-600	0.40 (0.12)	OZ03-1600	0.18 (0.08)	UBC227-1300	0.12 (0.06)
OF20-750	0.13 (0.06)	OZ04-575	0.85 (0.08)		

Population Integrity

Marker frequencies in cultivated production types (exotic and native) and wild populations of American ginseng, except populations P5 and P6, were significantly different ($P < 0.0001$) (Table 8). Plants cultivated from exotic seed were the most highly differentiated from the other populations and the combined Fisher's overall χ^2 values were greater than 500 for comparisons between exotic and native plants as well as between exotic plants and wild populations P1-P4. In comparison, the magnitude of the difference between the exotic production type and wild population P7 was low ($\chi^2 = 141$).

Table 8. Estimates of population differentiation based on marker frequency differences among all pair-wise comparisons of wild populations (P1-P7) and cultivated production types (exotic and native) of American ginseng grown in Maryland. Above the diagonal: Combined Fisher's overall significance values, below the diagonal: Combined Fisher's overall χ^2 values.

		P1	P2	P3	P4	P5	P6	P7	Exotic	Native
Wild:	P1		*	*	*	*	*	*	*	*
	P2	278		*	*	*	*	*	*	*
	P3	267	147		*	*	*	*	*	*
	P4	221	171	246		*	*	*	*	*
	P5	299	333	267	406		n.s.	*	*	*
	P6	221	193	147	297	73		*	*	*
	P7	423	442	376	566	208	200		*	*
Cultivated:	Exotic	504	573	532	596	334	334	141		*
	Native	238	273	205	277	244	159	390	499	

n.s. = non significant.

* Significantly different at $P < 0.0001$.

DISCUSSION

At the species level, cultivated and wild American ginseng grown in Maryland were genetically diverse. A large range of diversities has been reported for American ginseng in cultivated field plots, although all estimates have been considered high. Total genetic diversity of cultivated populations of native and exotic plants in Maryland ($SM = 0.27, 0.37, H_{t\beta} = 0.33, 0.38$) was high and within the range of diversity reported for cultivated populations of American ginseng ($SM = 0.20$ to 0.31) (Boehm et al., 1999; Schluter and Punja, 2002) (H_e [pooled] = 0.31) (Grubbs and Case, 2004). However, my estimates based on Jaccard's coefficient were lower ($JD = 0.37, 0.47$) than reported by Bai et al. (1997) ($JD = 0.59$). Differences in diversity estimates may in part be explained by the number of markers used; Bai et al. (1997) utilized 161 polymorphic markers and reported high levels of diversity. However, Boehm et al. (1999) used 82 RAPD markers to estimate genetic distance among *P. quinquefolius* plants, considerably more markers than used in this study (38 markers) or by Schluter and Punja (2002) (35 markers), but theirs was the lowest estimate of diversity reported for cultivated American ginseng ($SM = 0.20$). Genetic diversity is more likely influenced by the geographic range of the sources of seed cultivated in field plots as well as the degree of drift and strength of selection in these plots. Like previous studies, genetic diversity in cultivated field populations in Maryland was high and field plots appear to be collections of unimproved landraces that have not lost significant diversity via drift or selection. In addition, plants allegedly cultivated from native seed sources appear to be less diverse than plants cultivated from exotic commercial seed, which may reflect a local, more geographically limited, seed source

range as compared to the exotic seed which was derived from geographically widespread sources.

Total genetic diversity of wild ginseng was high ($SM = 0.33$, $H_{i\beta} = 0.33$) and comparable to the estimates reported by Boehm et al. (1999) ($SM = 0.24$) and Schluter and Punja (2002) ($SM = 0.27$). My estimate of total genetic diversity was lower than the estimate reported by Grubbs and Case (2004) (H_e [pooled] = 0.47) probably because populations in this study were sampled from a more limited geographic area. Although total diversity was high, wild populations in Maryland exhibited a large range of within-population diversities. Mean simple-matching genetic distance ranged from 0.12 to 0.33 in previous studies (Boehm et al., 1999; Schluter and Punja, 2002), whereas mean genetic distance within wild populations in Maryland ranged from 0.03 to 0.42. Low levels of diversity have been reported within wild ginseng populations in other parts of its range; however, conclusions were based on estimates of expected heterozygosity and homozygote excess (inbreeding) calculated from co-dominant allozyme markers (Cruse-Sanders and Hamrick, 2004; Grubbs and Case, 2004). In this study the analog of Nei's expected heterozygosity ($H_{e\beta}$) was lower in wild populations in Garrett County ($H_{e\beta} = 0.11$) than expected for mixed-mating species based on allozyme markers (H_e [at polymorphic loci] = 0.22; same as H_s in (Hamrick and Godt, 1989) and RAPD markers ($H_e = 0.22$) (Nybom and Bartish, 2000). Both genetic and phenological data suggest that American ginseng employs a mixed-mating system (Schlessman, 1985; Schluter and Punja, 2000; Schluter and Punja, 2002), but that self-pollination may be more prevalent than outcrossing (Grubbs and Case, 2004; Schluter and Punja, 2000).

Mean $H_{e\beta}$ for Garrett County populations (0.11) was similar to estimates of H_e reported for self-pollinating species ($H_e = 0.09$) (Nybom and Bartish, 2000) ($H_s = 0.15$) (Hamrick and Godt, 1989); however, $H_{e\beta}$ for Washington County populations (0.31) was greater than predicted for outcrossing species ($H_e = 0.26$) (Nybom and Bartish, 2000) ($H_s = 0.24$) (Hamrick and Godt, 1989).

RAPD fingerprints provided sufficient genetic data to distinguish exotic commercial plants from plants grown from native seed sources. Two groups, categorized as “native” and “exotic” in this study, were clearly distinguished in the spatial distribution of populations and individuals in PCA and MDS space. These spatial distributions were based on differences in marker frequencies between these two groups, as well as the presence of novel markers in exotic commercial plants. Wild populations in Garrett County (P1-P4) and plants cultivated from native seed (grown from seed putatively collected in wild populations in Garrett County) appeared genetically similar and formed a group with the greatest potential for being native. Mean simple-matching genetic distance among native cultivated plants and wild populations P1-P4 was lower (0.24-0.32) and the distance among exotic cultivated plants and P1-P4 higher (0.51-0.66) than previously reported among cultivated and wild populations in Wisconsin and Canada (Schluter and Punja, 2002) (0.43). These results support the idea that the majority of cultivated plants in this study were grown from locally-collected seed as previously thought (pers. comm. Trumbule) and that these plants were clearly distinct from exotic plants cultivated in Maryland. Unlike wild American ginseng grown in Pennsylvania, which had been reported to be similar to Wisconsin commercial ginseng (Boehm et

al., 1999), wild American ginseng in Garrett County, Maryland (P1-P4) was distinct from Wisconsin and Tennessee commercial ginseng. However, some wild Maryland populations did not appear to be native and were more genetically similar to exotic plants than those considered native.

Prior to this study, exotic plants grown in Maryland were only thought to exist in cultivated field plots. However, my data suggest that exotic germplasm is present in wild American ginseng populations in Maryland as well. The majority of individuals in population P7 grouped with plants grown from exotic seed. Exotic plants and individuals in wild population P7 possessed four common markers at high frequency that were largely absent from all other populations. Mean genetic distance between population P7 and exotic plants ($SM = 0.43$) was almost as large as between population P7 and native plants ($SM = 0.47$). However, the large distance between P7 and exotic plants was in large part caused by the high levels of diversity within population P7 ($SM = 0.42$) and exotic plants ($SM = 0.37$). Thus, the diversity within and among these overlapping exotic groups was approximately equal. In addition, there were four individuals in population P7 that were genetically distinct from exotic plants and instead were similar to native plants. These data suggest that exotic seed has been artificially introduced into at least one wild, formerly native population in Washington County. Wild populations P5 and P7 shared an additional four markers found predominantly in exotic germplasm; however, these markers were found in lower frequencies in P5 and this population was more closely related to native plants ($SM = 0.36$) than exotic plants ($SM = 0.49$). Thus, it remains unclear whether P5 contains a low frequency of introduced exotic individuals, has experienced gene flow

from neighboring exotic populations or whether native Washington County populations share some novel markers with exotic plants because of historic gene flow and migration between contiguous populations prior to harvest and habitat fragmentation. Regardless, my data suggest that in areas where wild harvest is prevalent, cultivated American ginseng germplasm may be mixed with native wild germplasm (Boehm et al., 1999).

All populations, wild and cultivated, were genetically distinct from one another with the exception of P5 and P6, which may be subpopulations. Plants cultivated from native seed, although similar to wild populations in Garrett County, were distinct. Differentiation likely resulted from both drift and differential selection at cultivated and wild sites as well as limited gene flow among wild and cultivated populations. Estimates of genetic structure among wild American ginseng populations in this study were higher ($G_{st\beta} = 0.41$) than would be predicted on average for a mixed-mating dicot ($G_{st} = 0.24, 0.19$) and more comparable to estimates for self-pollinating species ($G_{st} = 0.45, 0.59$) based on allozyme and RAPD markers, respectively (Hamrick and Godt, 1996; Nybom and Bartish, 2000). My results, like those of Grubbs and Case (2004) ($G_{st} = 0.63; \theta = 0.78$) indicated that genetic structure was much higher in wild American ginseng populations than reported by Schluter and Punja (2002) ($G_{st} = 0.28$). Grubbs and Case suggested that high levels of drift and inbreeding observed in their study were best attributed to a high degree of self-pollination in these populations; however, many factors influence genetic structure such as mating strategy, drift and population isolation. According to Cruse-Sanders and Hamrick (2004) wild American ginseng populations protected from harvest

disturbance were more diverse than unprotected populations and that genetic structure was lower among protected populations ($G_{st} = 0.17$) than unprotected populations ($G_{st} = 0.49$). Genetic structure was high ($G_{st\beta} = 0.41$) among Maryland populations, which is what was predicted given that harvest is legal in Maryland. My results were more similar to those reported by Cruse-Sanders and Hamrick (2004) ($G_{st} = 0.49$) for wild populations subject to harvest than those reported by Schluter and Punja (2002) ($G_{st} = 0.28$) for wild populations in Canada where harvest is illegal. Therefore, my results corroborate earlier findings that genetic structure is high and within-population diversity low in wild ginseng populations subject to harvest.

Estimates of θ_β in this study were estimated globally and for each marker separately. The majority of markers exhibited levels of genetic structure that fell within the confidence intervals of the global estimate; however, eight markers exhibited much lower levels of genetic structure ranging from 0.10 to 0.13. These were the same markers that were found in high frequency in population P7 and in exotic plants, but were largely absent from native germplasm. These results along with earlier findings that populations in Garrett County were genetically distinct from those in Washington County lead us to consider populations in each of these counties separately. When considered separately, genetic differentiation among populations in Garrett County (P1-P4) increased slightly ($G_{st\beta} = 0.52$), but decreased drastically among populations in Washington County (P5-P7) ($G_{st\beta} = 0.11$). These results might suggest that populations in Washington County were less influenced by harvest than those in Garrett County (Cruse-Sanders and Hamrick, 2004) ($G_{st} = 0.11$ vs. 0.17). However, a more likely scenario is that estimates of within-population diversity ($H_{e\beta}$)

were high and genetic structure ($G_{st\beta}$) low in populations in Washington County, because exotic commercial seedstocks from a variety of origins were introduced into these populations making them more similar to one another. However, the data in this study were not sufficient to preclude the possibility that populations in Washington County were experiencing higher levels of gene flow than in Garrett County due to natural causes.

CONSERVATION IMPLICATIONS

The combined effects of population isolation, drift and genetic contamination may threaten both the current fitness and future evolutionary potential of American ginseng populations in Maryland. Reductions in population size and gene flow caused by anthropogenic disturbances such as habitat fragmentation and harvest, as well as natural processes, can increase genetic drift and decrease genetic variation in wild populations (e.g. Ellstrand and Elam, 1993; Frankham, 1995). A significant correlation has been shown between population size and heterozygosity in wild American ginseng populations (Grubbs and Case, 2004), with smaller populations exhibiting lower levels of genetic diversity. The largest estimated population size was 60 plants for wild populations surveyed in this study, well below the minimum viable population size of 172 plants estimated by (Nantel et al., 1996). In Maryland, very low levels of diversity (S_M , $H_{e\beta}$, P) were estimated for two of the four native populations in Garrett County (P2, P4) and all populations were highly genetically differentiated ($G_{st\beta}$). Given these data, genetic factors such as inbreeding depression (Charlesworth and Charlesworth, 1987; Fischer and Matthies, 1998; Frankham, 1995; Hedrick and Kalinowski, 2000; Husband and Schemske, 1997; Keller and Waller,

2002) and loss of adaptive variation (Charlesworth and Charlesworth, 1987; Frankham, 1995) as well as demographic factors such as stochastic events (Lande, 1988), Allee effects (Hackney and McGraw, 2001; Lande, 1988) and loss of natural colonization events (Lande, 1988) increase the risk of extinction of some wild Maryland American ginseng populations.

Whereas inbreeding often has negative fitness consequences, outbreeding can provide a mechanism for recovery from inbreeding and for the creation of new diversity. Artificial seeding practices can be used to reduce the risk of extinction in small, inbred populations by increasing population sizes and opportunities for outbreeding. However, the introduction of non-native seed (often derived from cultivated field plots) into native populations may instead have detrimental fitness consequences, especially when plants are adapted to their local environments (Allendorf et al., 2001; Ellstrand and Elam, 1993). Intraspecific hybridization of native and exotic plants may lead to outbreeding depression: reductions in progeny fitness due to mal-adaptation to the local environment or the breakdown of co-adapted gene complexes in hybrid progeny (Lynch, 1991; Parker, 1992; Templeton, 1986; Waser, 1993). Local adaptation is most probable in species such as American ginseng that are highly self-compatible and experiencing little gene flow among populations (Ellstrand and Elam, 1993; Parker, 1992). The potential existence and threat of artificial introduction of non-native seed into wild American ginseng populations has been discussed (Cruse-Sanders and Hamrick, 2004; Grubbs and Case, 2004). However, this is the first study to provide evidence of two genetically distinct groups, native and introduced, growing in the same region. Although the biology and

genetic structure of ginseng populations suggest that intraspecific hybridization will potentially lead to negative fitness effects, this assumption has not yet been tested empirically in this system. My results demonstrate the need for such a study.

MANAGEMENT RECOMMENDATIONS

Total genetic variation in Maryland American ginseng was high and native germplasm was genetically distinct from exotic commercial germplasm. However, wild American ginseng populations surveyed in Maryland were small and highly genetically differentiated; some populations were likely introduced and genetic diversity was low in other populations. Loss of genetic resources not only has direct fitness consequences in wild populations, but also limits the resources available for the development of economically viable alternatives to unregulated wild harvest, most notably, reintroduction efforts and the development of improved cultivars. To reduce the loss of genetic diversity in these populations, further limits to harvest may be needed; however, American ginseng is one of the most valuable non-timber forest products available and wild harvest of ginseng yields significant revenue in rural communities. Thus, improved management practices (i.e. population census and monitoring programs, reintroduction and supplementation) would be a more viable solution than market closure.

Creation of a native seed bank would provide a mechanism to conserve genetic variation *ex situ* and provide a much-needed source of native seed. Sampling large amounts of seed from only a few populations is not likely to provide an adequate representation of the variation present in American ginseng germplasm because wild populations in Maryland are highly differentiated from one another.

Thus, entries should be selected from as many wild populations as possible. Ideally, the seed bank should be extended beyond political boundaries (i.e. state or county) to encompass regions of genetic or ecological similarity; therefore, sampling beyond state boundaries is recommended. Cultivated field populations represent an important source of seed for the collection because they are diverse and appear to be mixes of native lineages, but selection of both wild and cultivated seed entries should be based on genetic testing to clearly distinguish exotic seed from native. When introduced into native populations, exotic seed poses a serious threat to the protection of local variation and population viability. Only native seed should be used for future supplementation of wild Maryland populations. The RAPD fingerprints developed in this study provide an initial method for distinguishing some exotic sources. Once the collection is created, native seed should be utilized by plant breeders for genetic improvement and by collectors for supplementation of wild populations. Thereby minimizing dependence on exotic commercial seed and reducing the threat of widespread genetic contamination in Maryland American ginseng.

Chapter 3: Phytochemistry

ABSTRACT

American ginseng (*Panax quinquefolius* L.) is a highly-valued medicinal herb that is threatened by habitat loss (deforestation) and over-harvest and has been reported to occur primarily as geographically and genetically isolated patches. Current attempts to protect diversity in wild populations and to improve cultivation practices are restricted by the paucity of data available comparing the phytochemical diversity of wild and cultivated populations of American ginseng. The relative concentrations of five major ginsenosides (Rg1, Re, Rb1, Rc and Rd) were measured in wild and cultivated American ginseng root samples collected in Maryland. Significant variations in ginsenoside concentration and ginsenoside composition were observed among populations in Maryland. The major ginsenosides in American ginseng roots sampled in Maryland differed by seed source (native versus exotic). The main constituent in exotic chemotypes was Re (low Rg1/high Re) and the main constituent in native chemotypes was Rg1 (high Rg1/low Re). Roots exhibiting exotic chemotypes were observed in wild populations, suggesting that exotic germplasm had been introduced into wild populations in Maryland. Roots with similar chemotypes clustered based on a RAPD analysis of genetic similarity, indicating that variation in chemotype may be under genetic control. Thus, chemotype could provide a reliable indicator of the authenticity of wild Maryland American ginseng roots.

INTRODUCTION

American ginseng (*Panax quinquefolius*) is a perennial herb that grows in the understory of mature deciduous forests in mountainous regions of the eastern U.S. and Canada and is prevalent throughout the Appalachian Mountain region where it is native in Maryland. Asian ginseng (*Panax ginseng*) root has been used as a curative or tonic in Asian medicine for thousands of years and is very valuable. American ginseng is an equally valuable medicinal herb and for over 300 years it has been harvested in North America for export to Asia. Cultivation of American ginseng began in the late 1800s, but cultivated roots are considered less medicinally potent than wild roots (Persons, 1994). Hence, wild roots remain significantly more valuable than cultivated roots. Both wild harvest and cultivation of American ginseng create significant revenue in rural communities and provide economically viable alternatives to timber harvest. Unfortunately, wild harvest is not likely to be sustainable given the high demand for ginseng root, size-selective harvest (preferential collection of the largest or oldest plants in a population) and species demographics (Charron and Gagnon, 1991; Gagnon, 1999; Nantel et al., 1996).

Habitat destruction (deforestation) and over-harvesting of plants are generally considered to be the main factors responsible for the decline in American ginseng populations across its range. Ginseng is vulnerable, imperiled or extirpated in approximately 70% of its native range (NatureServe, 2004). Given the potential for this species to become endangered it was listed under CITES in 1975. For export to remain legal under CITES regulation, the U.S. Fish and Wildlife Service (USFWS) must ensure that export is not detrimental to the survival of wild ginseng. Thus, the

USFWS, in collaboration with individual states, monitors wild ginseng populations and regulates ginseng harvest and export (Robbins, 1998). Harvest is legal in 19 states in the U.S. including Maryland, but there is concern that populations are continuing to decline because of the prevalence of illegal poaching and inadequate funding for surveying, monitoring and enforcing restrictions on harvest (Gagnon, 1999). Wild-simulated or woods-grown roots are grown under natural forest conditions and are thought to be more medicinally similar to wild roots than traditionally cultivated roots. Thus, wild-simulated cultivation could provide a more economically viable alternative to wild harvest than traditional cultivation and an incentive to conserve forests. Artificial seeding practices have been prescribed to increase population sizes in the wild and reduce the impact of harvest, but the introduction of non-native plants (often originating from cultivated field plots) into locally adapted populations may have negative fitness consequences (Anderson et al., 2002). The benefits of wild-simulated cultivation and artificial seeding could be improved if more data were available comparing the phytochemistry of American ginseng grown in the wild and in cultivated field plots.

The pharmacological effects of ginseng have been attributed primarily to a class of compounds called triterpenoid saponin glycosides (dammarene-type saponins), also known as ginsenosides. More than twenty ginsenosides have been isolated from ginseng roots and leaves and are classified into two main groups: the glycosides of 20(S)-protopanaxadiol (20[S]-dammar-24-ene-3 β , 12 β , 20-triol) (Rb1, Rb2, Rc, Rd, Rg3 and Rh2) and those of 20(S)-protopanaxatriol (6 α -hydroxy-20[S]-protopanaxadiol) (Re, Rf, Rg1, Rg2, Rh1 and R1) (Court, 2000; Tanaka, 1994)

(Figure 7). The main ginsenosides isolated from American ginseng are Rb1, Rc, Rd, Re and Rg1 (Court et al., 1996a; Court et al., 1996b; Li et al., 1996) and Rb1 and Re typically account for greater than 70% of the total ginsenoside extracted from cultivated American ginseng (Assinewe et al., 2003; Court et al., 1996b; Li et al., 1996; Wills et al., 2002).

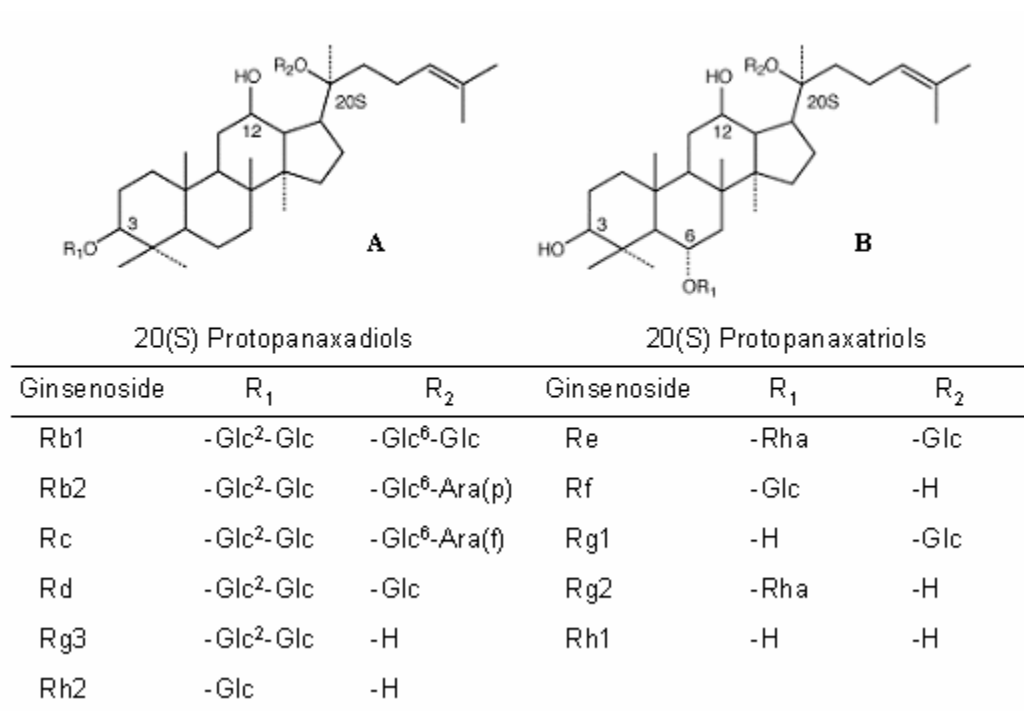


Figure 7. Chemical structures of two major groups of ginsenosides: (A) 20(S) Protopanaxadiols and (B) 20(S) Protopanaxatriols. Glc, glucose; Ara(p), arabinose in pyranose form; Ara(f), arabinose in furanose form; Rha, rhamnose; H, hydrogen. Content of figure is adapted from Attele et al. (1999) and Shibata (2001).

In general, the pharmacological effects associated with ginseng use include “adaptogenic” effects or the recovery of homeostasis (maintenance of chemical and metabolic equilibrium), effects on the central nervous system (CNS) (stimulatory or sedative effects, improved memory and learning), cardiovascular effects (lowering

blood pressure), antipsychotic effects, stress alleviation, improved gastrointestinal motility, immunological effects (i.e. inhibition of tumor growth) and enhancement of sexual behavior (Sticher, 1998; Tanaka, 1994).

Significant variability in the ginsenoside concentration of cultivated American ginseng roots has been reported (~2-20% total ginsenoside) (Attele et al., 1999) which has been attributed to the effects of age (Court et al., 1996a; Smith et al., 1996; Wills et al., 2002), dry weight (Wills et al., 2002), environmental conditions (i.e. soil fertility, light) (Fournier et al., 2003; Park and Lee, 1993) and population (Assinewe et al., 2003; Li et al., 1996). However, the relative ranking of the main ginsenoside constituents of cultivated American ginseng has been reported to differ very little (Assinewe et al., 2003; Court et al., 1996a; Li et al., 1996; Wills et al., 2002).

Ginsenosides isolated from American ginseng include: Rb1, Rb2, Rc, Rd, Re, Rg1 and Ro (Court et al., 1996a; Court et al., 1996b; Li et al., 1996) and the main constituents extracted are Rb1 and Re (Assinewe et al., 2003; Court et al., 1996a; Li et al., 1996; Wills et al., 2002). American ginseng is most easily distinguished from Asian ginseng by the absence of the ginsenoside Rf and less consistently by the ratio of Rg1:Rb1 (Assinewe et al., 2003; Chuang et al., 1995; Harkey et al., 2001).

American ginseng is noted for being the only species to have Rg1:Rb1 ratios of less than 1.0 (~0.15); however, when cultivated and wild American ginseng are considered separately, wild roots may contain much higher levels of Rg1, making this ratio an unreliable indicator of species (Attele et al., 1999; Chuang et al., 1995).

There are few studies of root ginsenoside variability in wild American ginseng populations. Wild populations are particularly difficult to study because sample sizes

are limited by the rarity of the species and because the locations of wild populations are often unknown or carefully guarded to protect populations from poachers. In the first and only comprehensive study of wild American ginseng in the U.S. and Canada, variability of total root ginsenoside was found to be high, ranging from 1-16% by dry weight (w/w), with the majority of roots containing 4-5% ginsenoside (Assinewe et al., 2003). Ginsenoside concentrations varied significantly in several of the wild populations (Assinewe et al., 2003). In addition, wild roots contained higher levels of Rg1 than reported for cultivated American ginseng roots. These authors speculated that the high level of phytochemical variation observed among these wild populations might be related to the high level of genetic isolation reported among wild American ginseng populations (Cruse-Sanders and Hamrick, 2004; Grubbs and Case, 2004).

In this study, the effect of population on ginsenoside concentration (% w/w) and ginsenoside composition (% total) was investigated. In Maryland, many ginseng growers cultivate plants from seed that allegedly has been collected from wild Maryland populations over the last few decades. However, little is known about the phytochemical diversity harbored in wild and cultivated American ginseng populations in Maryland. These data are important for authenticating the phytochemical profiles of wild and cultivated American ginseng for quality assurance programs, for verifying the phytochemical relatedness of plants grown in the wild and in cultivated field plots and for establishing the level of phytochemical variation harbored in Maryland populations. Knowledge of the phytochemical variation in Maryland ginseng populations will enable selection of improved cultivars based on chemical superiority and help guide the strategic development of a native seed bank.

Both of these outcomes have the potential to improve management practices and minimize the impacts of wild harvest in Maryland.

MATERIALS AND METHODS

Plant Material

Mr. Robert Trumbule, Plant Protection Specialist at the Maryland Department of Agriculture, collected forty-four roots for this study. Sixteen roots were collected from 5 wild populations and four Maryland growers donated 26 roots from five cultivated field plots (Table 9). Farms were located in four Maryland counties, F1 in Allegheny County, F2 and F3 in Garrett County and F4 in Frederick County. However, each farm was categorized by the putative origin of the seed being grown in the sampled field plot. The code MD denotes a native Maryland seed source and the code EX denotes an exotic seed source (Tennessee or Wisconsin seed). Roots grown from exotic seed sources on Farm 2 provided a control group against which putatively native roots were compared. Sample sizes for explanatory variables (population, age and dry weight) were difficult to control and root availability was limited because of rarity and limits to harvest. Generally, wild roots were older and smaller than cultivated roots (Table 9). In addition, only two wild samples were collected in 2002 because a drought caused plants to senesce early in the growing season.

Table 9. Population code, seed source, year collected, sample size (N) and mean age and dry weight of American ginseng roots collected in wild and cultivated populations in Maryland. Standard deviations are in parentheses.

Population	Seed Source	Year	N	Mean Age	Mean Dwt^a
Cultivated:				years	grams
F1MD	Allegheny County	2003	2	6.0 (2.8)	1.5 (1.1)
F2EX	TN, WI ^b	2002-2003	7	4.3 (0.8)	3.3 (2.3)
F2MD	Garrett County	2002-2003	3	6.7 (1.2)	2.7 (0.9)
F3MD	Garrett County	2002-2003	12	7.3 (1.0)	4.4 (3.0)
F4MD	Garrett County	2003	2	6.0 (0.0)	0.9 (0.2)
Total			26	6.2 (1.6)	3.4 (2.6)
Wild:				years	grams
P1WD	Garrett County	2003	2	4.0 (0.0)	0.6 (0.2)
P3WD	Garrett County	2002-2003	6	8.3 (1.5)	0.8 (0.7)
P5WD	Washington County	2003	3	6.0 (0.0)	0.9 (0.2)
P7WD	Washington County	2002-2003	4	8.0 (2.0)	1.0 (0.4)
P9WD	Allegheny County	2003	3	8.7 (2.3)	0.8 (0.1)
Total			18	7.5 (2.1)	0.9 (0.4)
Grand Total			44	6.7 (1.9)	2.4 (2.3)

^a Dwt = root dry weight.

^b TN = Tennessee and WI = Wisconsin.

Extraction of Ginsenosides

Roots were freeze-dried for 72 hrs, ground in a Wiley Mill (20 mesh) (Foss Tecator AB, Höganäs) and kept at room temperature until extraction. My methods were based on the protocol of Li et al. (1996), but were simplified and modified for the extraction of smaller samples. An accurately weighed sampled (100 mg) was transferred to a 25 ml Erlenmeyer flask. Ginsenosides were extracted in 5 ml of 80% aqueous methanol in a 70°C water bath for 1 hour. During extraction, samples were

stirred continuously and every 15 minutes flasks were removed from the water bath and vortexed briefly. Flasks were capped but vented to reduce pressure and minimize evaporation. Extracts were centrifuged for 5 min (5000 rpm, Sorvall® SA-600 rotor) (Sorvall® Inc., Newtown) and filtered using a 0.45-micron filter (Fisher Scientific International Inc., Hampton). Extracts were concentrated to 400 µl under a stream of N₂ and resuspended in 1.6 ml 100% Methanol. Samples were re-filtered and 20 µl of extract was immediately injected in the HPLC system.

HPLC Analysis

Ginsenosides were analyzed using a HP1100 high-performance liquid chromatography (HPLC) system (Agilent Technologies, Inc., Palo Alto) with gradient elution and a µBondapak® C18 reverse-phase column (10 µm, 4.6 mm x 150 mm) with a µBondapak™ C18 Sentry™ Guard column (10 µm, 3.9 mm x 20 mm) (Waters, Inc., Milford). The binary gradient employed the mobile phases (A) water and (B) acetonitrile (HPLC grade; Fisher Scientific International, Inc., Palo Alto) with a flow rate of 1.2 ml min⁻¹ according to the profile adapted from Court et al. (1996b): 0-20 min, 20-21% B; 20-25 min, 21-26% B; 25-29 min, 26-27% B; 29-43 min, 27-34%; 43-47 min, 34-36% B; 47-54 min, 36-43% B; 54-55 min, 43-95% B; 55-59 min 95% B and the diode array detector was set at 203 nm. Ginsenoside concentrations for each sample were calculated using respective standard curves ($r^2 > 0.99$ for each of the five ginsenosides tested) based on standards purchased from Sigma-Aldrich Fine Chemicals (Milwaukee) and Indofine Chemical Company Inc. (Hillsborough). Samples were tested in duplicate. Sample order was randomized but modified such that no two replicates were tested in a single day.

DNA Isolation

DNA was isolated from approximately 20 mg root powder following the standard DNeasy® Plant Mini Kit protocol (Qiagen, Valencia). DNA was eluted with 150 µl DNeasy AE buffer twice and the DNA concentration was estimated spectrophotometrically (A_{260}). DNA concentrations did not differ significantly and an aliquot of all samples was diluted 1:4 (5-10 ng µl⁻¹) for use in RAPD PCR. As recommended by Pandey et al. (1996), samples that did not amplify well were diluted until amplification improved.

RAPD PCR

Twenty RAPD decamers that produced 38 highly reproducible markers in Maryland American ginseng germplasm (see Chapter 2) were used in this study. Mixtures for RAPD PCR (25 µL) reactions contained 20 ng DNA, 10 mM KCL, 10 mM (NH₄)SO₄, 20 mM Tris-HCL, 3 mM MgSO₄, 0.1% Triton X-100, 0.1 mM dNTPs, 35 ng of a single decamer and 1 unit taq polymerase (NEB, Beverly). Amplification was performed on a GeneAmp® PCR system (Perkin-Elmer, Boston) or a Eppendorf Mastercycler Gradient (Brinkmann Instruments, Inc., Westbury) DNA thermocycler for 45 cycles according to the procedure of (Williams et al., 1990). Approximately 15 µL of the total PCR volume was loaded onto a 2 % agarose gel containing ethidium bromide (0.5 µg ml⁻¹) and PCR fragments were separated by electrophoresis for 2 hrs (120 V). RAPD fragments were illuminated under UV light and images were captured with an Eagle Eye II instrument using EagleSight software (Stratagene, La Jolla). All 38 RAPD markers were successfully amplified from 40 of

the 44 samples. The remaining four samples were removed from the genetic analysis because data were not adequately reproducible.

Data Analysis

Ginsenoside concentrations were expressed as the weight of ginsenoside relative to the dry root weight (% w/w). Ginsenoside composition was expressed as the concentration of each ginsenoside (Rg1, Re, Rb1, Rc or Rd) relative to the total ginsenoside in that sample (% total). Spearman's non-parametric rank correlations among ginsenoside variables (% w/w total ginsenoside, Rg1, Re, Rb1, Rc and Rd), were analyzed using PROC CORR in SAS (SAS Institute Inc., 2002).

One-way analyses of variance (ANOVA) were conducted using PROC GLM in SAS (SAS Institute Inc., 2002). Ginsenoside concentration and composition data were not normal ($W < 0.95$, $P < 0.05$) (Shapiro and Wilk, 1965); therefore, non-parametric methods were used for statistical analyses. Ginsenoside data were ranked (PROC RANK) and one-way analyses of variance (ANOVA) were conducted on the ranked data (PROC GLM) (SAS Institute Inc., 2002). Least significant difference (LSD) values were calculated to determine differences among population means. The LSD values were calculated at the $P = 0.05$ level to increase the power to detect real significant differences and at the $P = 0.01$ level to adjust the experiment-wise alpha level for multiple comparisons. Results were reported as arithmetic means with standard errors of the means and as rank-transformed means used to test for significant differences.

Simple-matching genetic distances were calculated for all pair-wise comparisons of root samples based on the RAPD marker haplotype expressed in each

root sample (Sneath and Sokal, 1973). Cluster analysis was conducted on the resulting 40 x 40 simple-matching genetic distance matrix. The Neighbor-Joining clustering algorithm (Saitou and Nei, 1987) was used to visualize genetically related groups of root samples in NTSYSpc (Rohlf, 1998).

RESULTS

Typical chromatograms of the two main ginsenoside chemotypes observed in this study as well as ginsenoside standards are shown in Figure 8. Chemotype (A) and (B) differed in the relative amounts of panaxatriol ginsenosides (Rg1 and Re) extracted from American ginseng roots. Chemotype (A) had a low level of Rg1 ginsenoside and a high level of Re ginsenoside, whereas chemotype (B) had a high level of Rg1 and a low level of Re. Both of these chemotypes were observed in wild and cultivated American ginseng roots samples collected in both years (2002/2003).

All individual root ginsenosides, except Re, were directly correlated with total ginsenoside, but differed in the magnitude of that correlation (Table 10). Panaxadiol ginsenoside concentrations (Rb1, Rc and Rd) were more highly correlated with total ginsenoside than were panaxatriol ginsenosides (Rg1 and Re) (Table 10). Rb1 was more highly correlated with total ginsenoside than Rc or Rd ginsenoside. Panaxadiol ginsenosides were all positively correlated with each other; in contrast panaxatriol ginsenosides (Rg1 and Re) were negatively correlated with each other (Table 10).

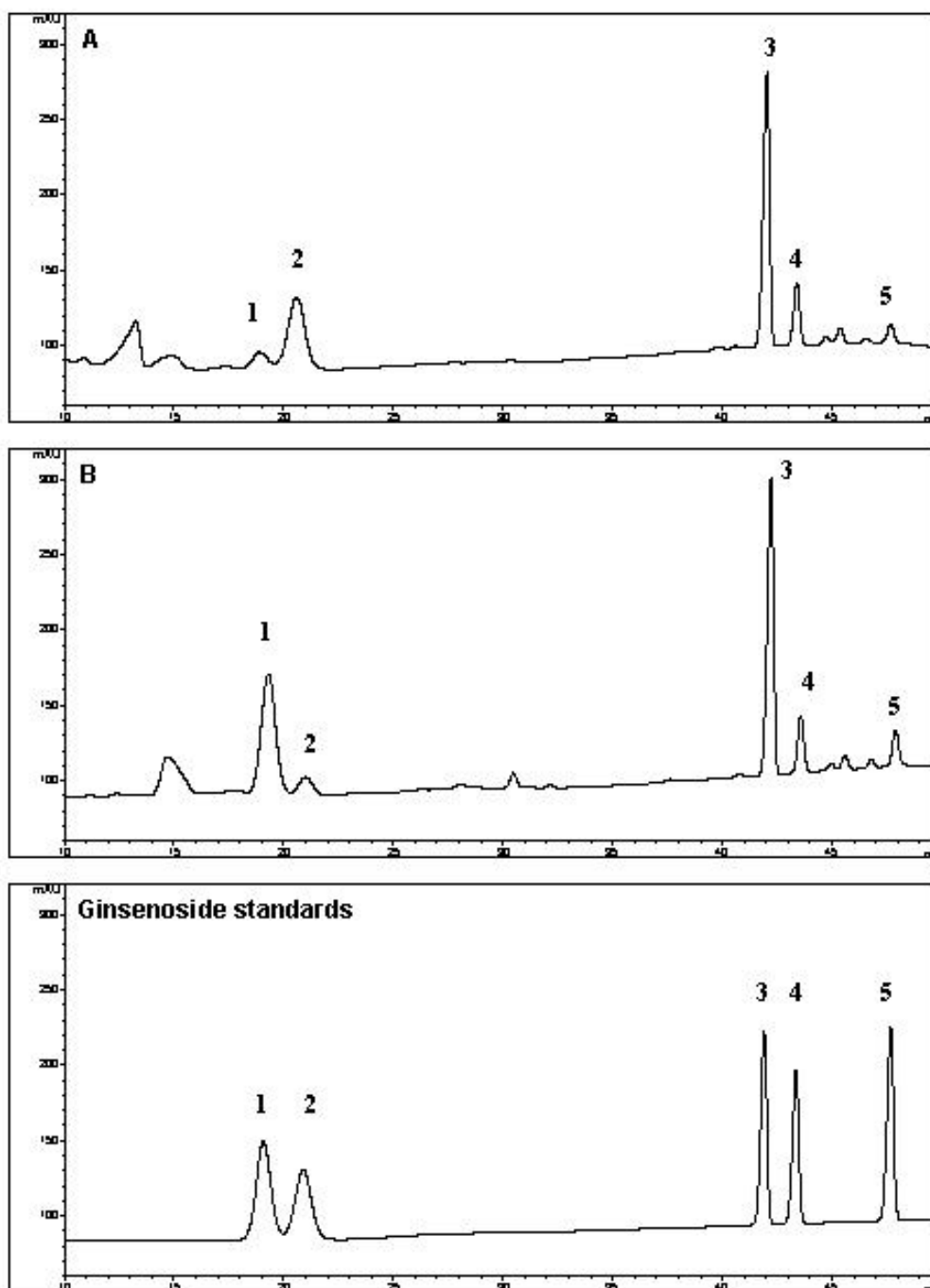


Figure 8. Typical HPLC chromatograms of ginsenosides observed in American ginseng roots collected in Maryland: peak 1 (Rg1), peak 2 (Re), peak 3 (Rb1), peak 4 (Rc) and peak 5 (Rd). Two main chemotypes were observed in this study, low Rg1/high Re (chemotype A) and high Rg1/low Re (chemotype B).

Table 10. Spearman's non-parametric rank correlations of root ginsenoside concentration for all 44 American ginseng samples collected in Maryland.

	Total	Rg1	Re	Rb1	Rc	Rd
Total	1.00	0.45**	0.20	0.96***	0.73***	0.57***
Rg1		1.00	-0.70***	0.40**	0.48**	0.30*
Re			1.00	0.18	-0.07	-0.03
Rb1				1.00	0.72***	0.60***
Rc					1.00	0.46**
Rd						1.00

* Significantly different at $P < 0.05$.

** Significantly different at $P < 0.01$.

*** Significantly different at $P < 0.001$.

Total ginsenoside concentration for each of the 44 root samples included in this study ranged from 0.85% to 5.78% with a mean of $2.35 \pm 1.04\%$ (median 1.96%). Total ginsenoside in cultivated roots ranged from 1.04% to 4.07% with a mean of $2.25 \pm 0.85\%$ (median 1.96%) and in wild roots ranged from 0.85% to 5.78% with a mean of $2.50 \pm 1.28\%$ (median 1.93%).

Ginsenoside concentration and composition frequency distributions for Rg1 and Re were bimodal. Therefore, for estimation of measures of central tendency, individuals were grouped based on their Rg1 and Re composition: low Rg1/high Re ($> 30\%$ Re, $\leq 20\%$ Rg1 and Re: $Rg1 > 2.0$) and high Rg1/low Re ($> 30\%$ Rg1, $\leq 20\%$ Re and Rg1: $Re > 2.0$). Based on this categorization technique, 41 out of 44 roots grouped into one of these two chemotypes: high Re or high Rg1. The remaining three roots exhibited intermediate chemotypes: high Re, but intermediate Rg1 ($20\% < Rg1 < 30\%$). In Table 11, ginsenoside concentrations and compositions are reported for each chemotype. Based either on ginsenoside concentration or on ginsenoside

composition, plants exhibiting a high Re chemotype (N = 17) were ranked: Re > Rb1 > Rg1 \cong Rc > Rd and plants exhibiting a high Rg1 chemotype (N = 24) were ranked: Rg1 > Rb1 > Re \cong Rc > Rd.

Table 11. Mean (\pm standard deviation) root ginsenoside concentrations and composition calculated for the two major chemotypes observed in American ginseng roots grown in Maryland. N = number of roots per chemotype.

Ginsenoside Concentration						
Chemotype	Rg1	Re	Rb1	Rc	Rd	Total
High Rg1 ^a	1.11 \pm 0.46	0.11 \pm 0.11	0.71 \pm 0.39	0.22 \pm 0.07	0.08 \pm 0.03	2.24 \pm 0.87
High Re ^b	0.22 \pm 0.17	1.05 \pm 0.43	0.89 \pm 0.67	0.20 \pm 0.10	0.08 \pm 0.03	2.45 \pm 1.29

Ginsenoside Composition					
Chemotype	Rg1	Re	Rb1	Rc	Rd
High Rg1 ^a	50 \pm 7	6 \pm 5	30 \pm 5	11 \pm 3	4 \pm 1
High Re ^b	9 \pm 5	46 \pm 8	33 \pm 8	9 \pm 2	4 \pm 2

^a High Rg1 = Rg1 >30%, Re \leq 20% (N = 24).

^b High Re = Re >30%, Rg1 \leq 20% (N = 17).

F-values from an ANOVA using rank-transformed ginsenoside concentration data are presented in Table 12. The F-values indicate the relative levels of difference among American ginseng populations for total and individual ginsenoside concentration. Differences among these populations grown in Maryland were significant for total ginsenoside and all individual ginsenosides, except Rd, and were highly significant for Rg1 and Re (Table 12). An ANOVA conducted on rank-transformed ginsenoside composition also showed significant differences among populations (Table 12). Differences among these populations were significant for all ginsenosides and highly significant for Rg1, Re and Rd (Table 12).

Table 12. Effects of population on American ginseng root ginsenoside concentrations and root ginsenoside compositions in Maryland.

Rank Ginsenoside Concentration							
F Value							
Source of Variation	dF	Rg1	Re	Rb1	Rc	Rd	Total
Population	9	9.25***	5.04***	3.60**	3.07**	1.58	3.71**

Rank Ginsenoside Composition							
F Value							
Source of Variation	dF	Rg1	Re	Rb1	Rc	Rd	
Population	9	10.70***	6.85***	3.33**	2.50*	7.63***	

ANOVA comparisons were based on ranked data (PROC RANK).

* Significantly different at $P < 0.05$.

** Significantly different at $P < 0.01$.

*** Significantly different at $P < 0.001$.

Means and standard errors of ginsenoside concentrations and compositions for wild and cultivated populations of American ginseng are presented in Tables 13 and 14. Significant differences among populations are presented in Tables 15 and 16.

Comparisons on the rank-transformed data were made based on the least significant difference (LSD) between populations at $P = 0.05$ ($LSD^{0.05}$). Wild population P5WD had the highest total (4.39%) and Rb1 (1.89%) ginsenoside concentrations, significantly greater than wild populations P1WD, P3WD and P9WD and cultivated populations F2EX, F2MD and F4MD (Total: 1.43-1.95%, Rb1: 0.41-0.58%).

Population P5WD had the highest Rb1 composition (42% of total), significantly greater than populations P3WD, P9WD and F2EX (27-28%). Rc and Rd ginsenoside concentrations varied little among populations. However, Rd composition was significantly greater in population F2EX (6.0%) than populations F1MD, F2MD, F3MD, F4MD, P5WD, P7WD and P9WD (3-4%).

Table 13. Mean ginsenoside concentrations and standard errors of the mean for cultivated and wild populations of American ginseng in Maryland. N = number of roots sampled per population.

Population	N	Ginsenoside Concentration					Total
		Rg1	Re	Rb1	Rc	Rd	
F2EX	7	0.13±0.09	0.76±0.03	0.41±0.11	0.14±0.03	0.09±0.03	1.53±0.32
F1MD	2	0.15±0.04	1.13±0.71	0.99±0.85	0.17±0.02	0.08±0.01	2.50±1.64
P7WD	4	0.21±0.11	1.21±0.50	1.11±0.45	0.29±0.12	0.06±0.02	2.87±1.03
P5WD	3	0.64±0.24	1.43±0.44	1.89±0.81	0.31±0.09	0.11±0.03	4.39±1.21
P9WD	3	0.62±0.39	0.46±0.55	0.53±0.03	0.24±0.08	0.06±0.01	1.90±0.09
P1WD	2	0.65±0.19	0.15±0.03	0.43±0.23	0.15±0.01	0.06±0.03	1.43±0.50
P3WD	6	0.89±0.48	0.18±0.15	0.56±0.35	0.24±0.08	0.08±0.03	1.95±1.00
F4MD	2	0.99±0.01	0.11±0.07	0.58±0.04	0.22±0.03	0.06±0.01	1.95±0.01
F2MD	3	1.06±0.36	0.07±0.09	0.56±0.22	0.17±0.06	0.06±0.03	1.92±0.72
F3MD	12	1.25±0.53	0.28±0.42	0.93±0.40	0.22±0.06	0.10±0.03	2.78±0.74

Table 14. Mean ginsenoside compositions and standard errors of the mean for cultivated and wild populations of American ginseng in Maryland. N = number of roots sampled per population.

Population	N	Ginsenoside Composition				
		Rg1	Re	Rb1	Rc	Rd
F2EX	7	8±2	51±2	27±1	9±1	6±1
F1MD	2	7±2	45±1	36±7	8±3	4±1
P7WD	4	7±2	43±4	38±3	10±1	2±0
P5WD	3	16±5	33±1	42±4	7±1	2±0
P9WD	3	33±12	23±16	28±1	13±3	3±0
P1WD	2	46±2	11±1	29±5	11±2	4±0
P3WD	6	46±3	9±2	28±1	13±1	4±0
F4MD	2	51±0	5±3	30±1	11±1	3±0
F2MD	3	56±5	3±2	29±3	9±1	3±0
F3MD	12	44±4	12±5	32±2	8±0	3±0

Table 15. Mean rank-transformed ginsenoside concentrations and significant differences among American ginseng populations in Maryland. N = number of roots sampled per population.

Population	N	Rank Ginsenoside Concentration					Total
		Rg1	Re	Rb1	Rc	Rd	
F2EX	7	5.9	29.7	10.7	8.2	26.5	10.4
F1MD	2	8.0	34.0	24.5	14.8	22.5	22.0
P7WD	4	9.1	37.8	33.3	30.5	14.6	30.5
P5WD	3	21.3	40.7	40.7	36.7	32.7	40.7
P9WD	3	22.3	22.3	18.3	27.7	11.0	20.3
P1WD	2	22.5	16.0	12.5	10.3	14.0	9.0
P3WD	6	26.5	16.0	16.1	28.0	22.6	17.7
F4MD	2	32.5	14.0	21.0	26.5	12.8	21.5
F2MD	3	32.0	10.7	16.7	16.3	13.2	15.7
F3MD	12	33.3	15.5	28.5	24.8	28.5	29.5
LSD ^{0.05}		12.5	15.2	16.6	17.3	19.5	16.5
LSD ^{0.01}		16.8	20.4	22.3	23.2	26.2	22.2

ANOVA comparisons were based on rank data (PROC RANK). Means within columns that differed more than the least significant difference (LSD) were significantly different at the given probability level of 0.05 and 0.01. The harmonic mean of the sample size was 3.2.

Mean Re composition, as well as Rd composition, was highest in F2EX (51%) and was significantly greater than populations F2MD, F3MD, F4MD, P1WD and P3WD (3-12%). Mean Re concentrations were highest in populations P5WD and P7WD (1.43 and 1.21%, respectively) and significantly greater than in populations P1WD, P3WD, P9WD, F2MD, F3MD and F4MD (0.07-0.46%). Mean Rg1 concentrations and compositions were significantly greater in populations P1WD, P3WD, F2MD, F3MD and F4MD (0.65-1.25%, 44-51% of total) than F2EX, F1MD and P7WD (0.13-0.21%, 7-8% of total).

Table 16. Mean rank-transformed ginsenoside composition and significant differences among American ginseng populations in Maryland. N = number of roots sampled per population.

Population	N	Rank Ginsenoside Composition				
		Rg1	Re	Rb1	Rc	Rd
F2EX	7	7.6	39.0	12.3	20.7	39.0
F1MD	2	8.0	34.5	30.0	17.0	20.5
P7WD	4	7.0	33.3	37.0	24.8	7.5
P5WD	3	14.7	27.7	40.3	9.7	6.0
P9WD	3	23.0	24.0	15.7	32.3	14.7
P1WD	2	27.0	20.0	18.5	30.5	32.5
P3WD	6	29.3	16.2	14.5	35.3	31.5
F4MD	2	35.5	13.5	21.0	32.5	14.5
F2MD	3	37.7	9.0	19.3	21.3	17.3
F3MD	12	30.5	14.1	25.3	15.3	20.8
LSD ^{0.05}		11.9	13.9	17.0	18.1	13.4
LSD ^{0.01}		16.0	18.6	22.8	24.2	18.0

ANOVA comparisons were based on rank data (PROC RANK). Means within columns that differed more than the least significant difference (LSD) were significantly different at the given probability level of 0.05 and 0.01. The harmonic mean of the sample size was 3.2.

Overall, plants cultivated from exotic seed (F2EX), differed significantly in Rg1 and Re ginsenoside composition from cultivated populations F2MD, F3MD and F4MD and wild populations P1WD and P3WD, but did not differ significantly from cultivated population F1MD and wild populations P5WD and P7WD. Rg1 and Re compositions in each cultivated and wild American ginseng population are displayed in Figure 9 and populations are categorized by chemotype. Populations F2EX, F1MD, P5WD and P7WD exhibited high Re chemotypes (> 30% Re, ≤ 20% Rg1), populations F2MD, F3MD, F4MD, P1WD and P3WD exhibited high Rg1

chemotypes ($> 30\%$ Rg1, $\leq 20\%$ Re) and population P9WD exhibited an intermediate chemotype ($> 30\%$ Rg1, $> 20\%$ Re).

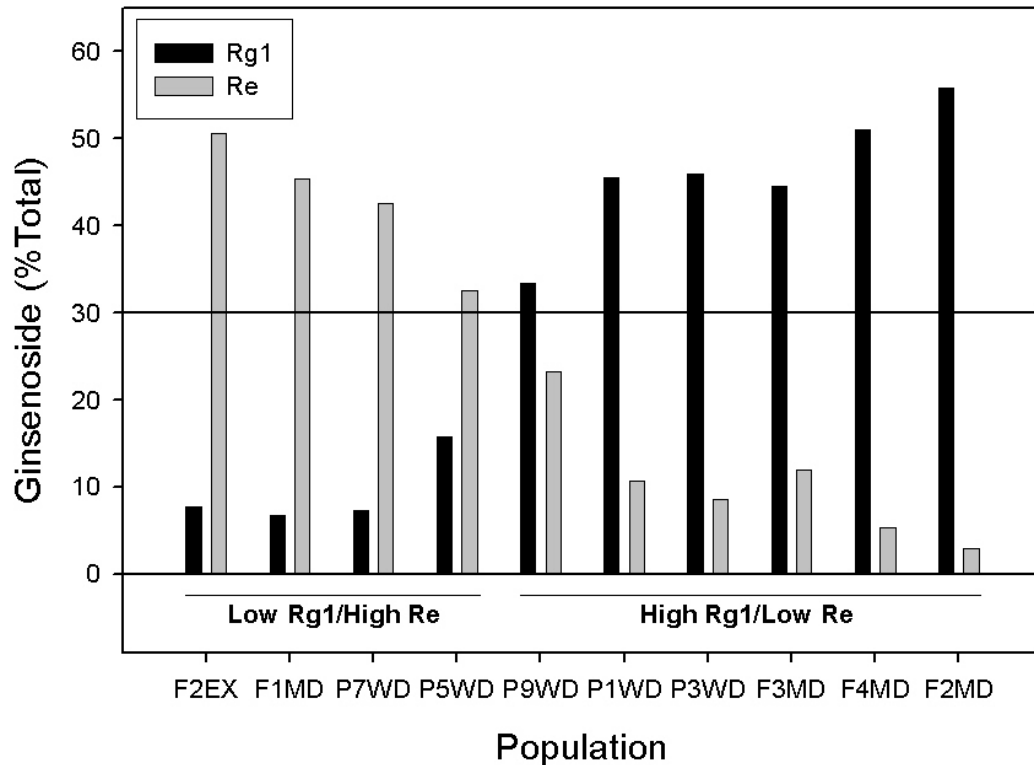


Figure 9. Rg1 and Re compositions in cultivated and wild populations of American ginseng in Maryland. Populations are categorized by chemotype: low Rg1/high Re ($> 30\%$ Re, $\leq 20\%$ Rg1) or high Rg1/low Re ($> 30\%$ Rg1, $\leq 20\%$ Re). A reference line was included at 30% ginsenoside composition to aid in chemotype visualization.

The frequency of roots that exhibited the high Rg1 ($> 30\%$ Rg1, $\leq 20\%$ Re) and high Re ($> 30\%$ Re, $\leq 20\%$ Rg1) chemotypes in cultivated and wild American ginseng populations in Maryland are presented in Table 17. Two roots from F3MD and one root from P5WD exhibited intermediate chemotypes and were not included in this analysis. Chemotype was highly associated with population but not propagation type (wild or cultivated). Populations F3MD and P9WD contained roots

exhibiting both chemotypes and the high Rg1 chemotype was predominant in both populations. Because both chemotypes were present in P9WD, this population possessed an intermediate chemotype (Figure 9). All roots in cultivated populations F1MD and F2EX and wild populations P5WD and P7WD possessed the high Re chemotype. All roots in cultivated populations F2MD, F3MD and F4MD and wild populations P1WD and P3WD possessed the high Rg1 chemotype.

Table 17. Number of roots that exhibited the high Rg1 and the high Re chemotypes in wild and cultivated American ginseng populations in Maryland. Roots that exhibited intermediate chemotypes were not included in this analysis.

Cultivated				Wild			
Pop	High Rg1^a	High Re^b	Total	Pop	High Rg1^a	High Re^b	Total
F2EX	0	7	7	P1WD	2	0	2
F1MD	0	2	2	P3WD	6	0	6
F2MD	3	0	3	P5WD	0	2	2
F3MD	9	1	10	P7WD	0	4	4
F4MD	2	0	2	P9WD	2	1	3
Total	14	10	24	Total	10	7	17

^a High Rg1/Low Re (Rg1 > 30%; Re ≤ 20%)

^b Low Rg1/high Re (Re > 30%; Rg1 ≤ 20%).

The low Rg1/high Re chemotype was associated with roots grown from exotic seed sources (F2EX) (Figure 9 and Table 17). The high Rg1 chemotype was associated with roots that were collected from wild populations in Garrett County, Maryland (P1WD and P3WD) or from field plots where native Maryland seed was putatively propagated (F2MD, F3MD and F4MD) (Figure 9 and Table 17). This is the first study to report a high Rg1/low Re chemotype in American ginseng roots.

However, not all roots allegedly grown from native Maryland seed exhibited the high Rg1 chemotype. Although the frequency of contamination differed, roots in three wild Maryland populations (P7WD, P5WD and P9WD) and one cultivated population (F1MD) exhibited exotic chemotypes.

A Neighbor-Joining cluster analysis based on the simple-matching genetic distance among individual ginseng roots supported the previously described phytochemical relationships among root samples (Figure 10). The two outermost clusters are labeled A and B and clusters of interest within A and B are labeled 1, 2 and 3 to aid in visualization. The majority of roots in cluster A exhibited native chemotypes. Exotic plants in populations F2EX and P7WD grouped together in cluster B. Cluster 1 grouped roots with native chemotypes together and separated them from four samples in cluster A (F1MD, F3MD and P5WD) that had exotic chemotypes. Although these individuals had exotic chemotypes they were more closely related to native than exotic plants based on their RAPD haplotypes. All of the plants in cultivated population F3MD, except one, were included in cluster 1, even though two of those roots had intermediate chemotypes. Clusters 2 and 3 grouped samples from P7WD separately from exotic plants (F2EX). An isolated sample from P5WD was included in cluster B, external to clusters 2 and 3. Thus, cluster analysis using genetic data clearly distinguished plants with exotic chemotypes (F2EX and P7WD) from plants with native chemotypes (F2MD, F3MD, F4MD, P1WD, P3WD and P9WD) with the exception of six individuals in P5WD, F1MD and F3MD that had exotic or intermediate chemotypes, but genetically were more closely related to plants with native chemotypes.

Population	High Rg1	High Re	ID
F3MD	INT	+	01
F1MD	-	+	16
F3MD	+	-	02
F3MD	+	-	04
F3MD	+	-	03
F3MD	+	-	23
F2MD	+	-	13
F2MD	+	-	14
P3WD	+	-	06
P3WD	+	-	29
P3WD	+	-	30
P3WD	+	-	31
P3WD	+	-	38
P3WD	+	-	39
F3MD	+	-	05
F3MD	INT	+	28
F3MD	-	+	24
F3MD	+	-	26
F3MD	+	-	25
F3MD	+	-	27
P9WD	+	-	18
P9WD	+	-	37
P1WD	+	-	21
P1WD	+	-	22
F4MD	+	-	19
F4MD	+	-	20
P5WD	-	+	32
P5WD	-	+	33
F2EX	-	+	07
F2EX	-	+	11
F2EX	-	+	12
F2EX	-	+	09
F2EX	-	+	10
F2EX	-	+	08
P7WD	-	+	36
P7WD	-	+	15
P7WD	-	+	35
P7WD	-	+	40
F1MD	-	+	17
P5WD	INT	+	34

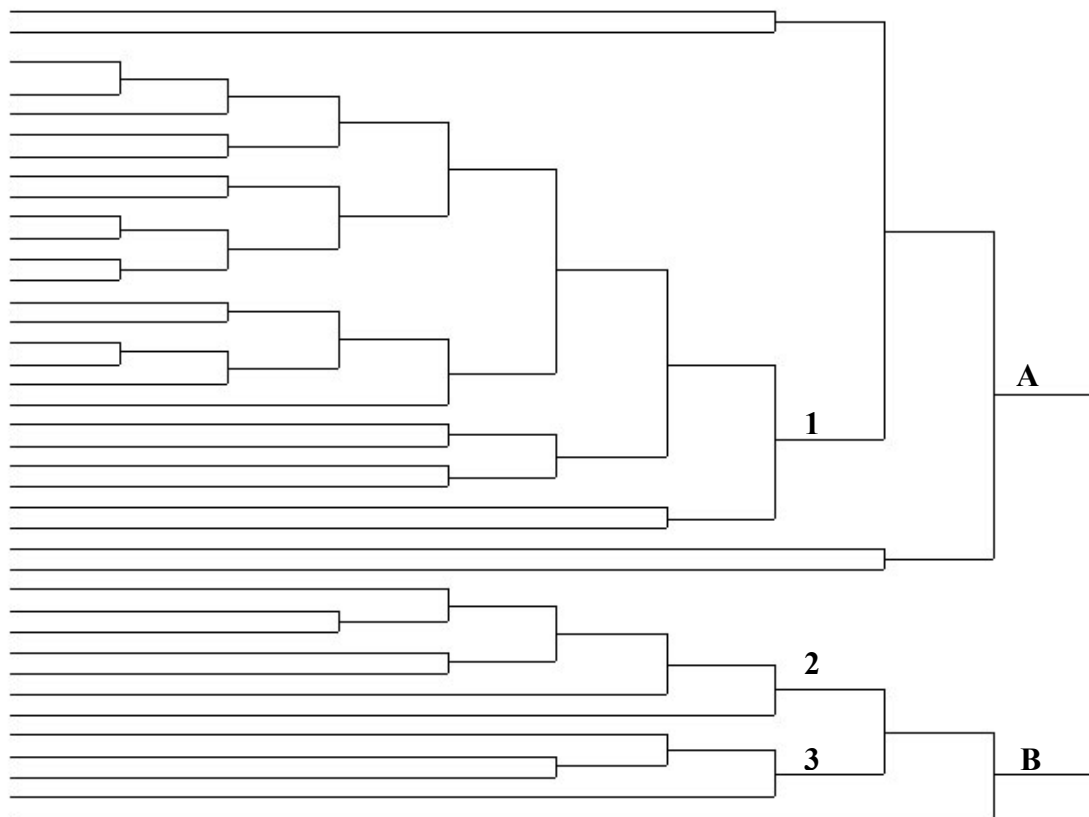


Figure 10. Relationships between RAPD type and chemotype visualized using a Neighbor-Joining cluster analysis of the simple-matching genetic distance matrix of 40 American ginseng roots. Symbols: '+' = High (> 30%), '-' = Low ($\leq 20\%$) and 'INT' = intermediate ($30\% \geq \text{INT} > 20\%$).

DISCUSSION

Significant variation in ginsenoside concentration and ginsenoside composition were observed in wild and cultivated American ginseng populations in Maryland. The major ginsenosides in American ginseng roots sampled in wild and cultivated populations in Maryland differed and the majority of root samples exhibited one of two chemotypes: high Rg1/low Re or low Rg1/high Re. Ginsenoside chemotype differed by seed source (native versus exotic) and the main constituents in the native chemotype were Rg1 (~45%) and Rb1 (~30%) and the main constituents in the exotic chemotype were Re (~45%) and Rb1 (~30%). Mixed chemotypes within a population or roots with intermediate chemotypes were rarely observed. Native chemotypes were observed in wild populations in Garrett County, but exotic chemotypes were observed in wild populations in Washington County. Thus, it appears that exotic plants have been artificially introduced into Washington County populations. Root samples that were genetically similar also generally shared the sample chemotype, consequently variation in chemotype may be governed more by genetic than environmental variability. Furthermore, chemotype may provide a good indicator for the authentication of wild Maryland roots.

Mean total ginsenoside concentration reported in this study was in the range reported for 4 yr old cultivated American ginseng (2-6%) (Court et al., 1996a; Li et al., 1996; Smith et al., 1996; Wills et al., 2002). Mean total ginsenoside in wild populations in Maryland was 2.50%, which was lower than 5.78% reported by Assinewe et al. (2003). Lower concentrations of total ginsenoside reported in this study were likely a factor of root age and extraction methodology. Roots aged 4-37

years were included in the study of Assinewe et al. (2003), but roots in my study ranged from 3-10 years of age. In addition, ginsenoside concentration was a relative measure in this study and percent ginsenoside recovery was less than 100%. Thus, relative ginsenoside concentrations must be compared cautiously between studies. In this study, total ginsenoside concentration differed significantly among populations ($P < 0.01$) and was greatest in population P5WD (4.39%), followed by populations P7WD (2.87%) and F3MD (2.77%). On average, roots in population P5WD were 6.0 years old as compared to 8.0 years old in P7WD and 7.3 years old in F3MD. Thus, for the roots sampled in these populations, age did not appear to be a major factor (see Appendix B for scatter plots of total ginsenoside by age class). However, the populations with the youngest plants, P1WD and F2EX (4.0 and 4.3 years) did have the lowest total ginsenoside concentrations (1.43% and 1.53%, respectively), suggesting that age may be a more important factor when roots are young. Total ginsenoside was similar between cultivated and wild populations (2.25% versus 2.50%, respectively). These results are consistent with the findings of Assinewe et al. (2003); however, there was not sufficient power to test this assumption in this study.

Rb1 ginsenoside concentrations varied significantly among populations ($P < 0.01$) and were highly correlated with total ginsenoside concentration suggesting that panaxadiol concentration, in particular Rb1, was a good indicator of total ginsenoside concentration. Rb1 concentrations, like total ginsenoside were lower in wild roots in this study than reported by Assinewe et al. (2002). On average, wild roots contained 0.88% Rg1 in this study as compared to 2.88% Rb1 ginsenoside (Assinewe et al., 2003). In this study, on average, Rb1 accounted for 35% of the total ginsenoside, but

in previous studies, Rb1 accounted for approximately 45% of the total ginsenoside (Assinewe et al., 2003; Court et al., 1996a; Li et al., 1996; Wills et al., 2002). Wild populations P5WD and P7WD exhibited exotic chemotypes and high Rb1 levels (Rb1 concentrations were 1.89% and 1.11% and accounted for 42% and 38% of the total root ginsenoside, respectively), but high Rb1 levels were not found in all populations exhibiting exotic chemotypes. Mean Rb1 composition for plants cultivated from exotic seed (F2EX) was 27%, similar to compositions estimated for native populations (P1WD, P3WD, F2MD, F3MD and F4MD). In addition, population F2EX had significantly higher compositions of Rd than P5WD or P7WD. These data suggest either that populations P5WD and P7WD are distinct from population F2EX or that Rb1 levels are at least partially under environmental control.

Mean Rg1 and Re concentrations and compositions differed significantly among populations ($P < 0.001$). In previous studies, Rg1 and Re concentrations varied little among cultivated populations, but Rb1, Rc and Rd ginsenoside concentrations varied significantly (Li et al., 1996, Wills et al. 2002). These findings indicated a strong genetic rather than environmental influence on Rg1 and Re concentrations. The majority of American ginseng roots and populations could be categorized into two distinct chemotypes based on Rg1 and Re composition: low Rg1/high Re and high Rg1/low Re. The high Re chemotype was similar to the profile reported previously for cultivated American ginseng (Court et al., 1996a; Li and Wardle, 2002; Li et al., 1996; Smith et al., 1996; Wills et al., 2002). Roots cultivated in Maryland from exotic seed (F2EX) as well as roots in populations F1MD, P5WD and P7WD exhibited this profile. The high Rg1 chemotype was unique to this study

and was observed in populations F2MD, F3MD, F4MD, P1WD, P3WD and P9WD. In a few cases, either both chemotypes (high Rg1 and high Re) were found within a single population (F3MD, P9WD) or populations contained roots that had high Re, but intermediate Rg1 compositions (F3MD, P5WD).

High levels of Rg1 have been reported for wild American ginseng samples previously, but were based on small numbers of wild samples of unknown origin (Awang, 2000; Wang et al., 1999) or were reported in conjunction with high levels of Re (Assinewe et al., 2003). The ginsenoside compositions reported for cultivated and wild American ginseng roots in four previous studies were compared to the results of this study (Table 18). The exotic chemotype in cultivated populations F1MD and F2EX was highly similar to the Rg1 and Re compositions previously reported for cultivated American ginseng roots. Since, wild populations P5WD and P7WD also exhibited exotic chemotypes, chemotype was not specific to production type (cultivated versus wild), but rather appeared to be specific to seed source (native versus exotic). Exotic plants in this study were grown from seed purchased from commercial field plots in Tennessee and Wisconsin. In earlier studies, plants were typically cultivated from Wisconsin or Canada seed sources (Assinewe et al., 2003; Court et al., 1996a; Li et al., 1996; Smith et al., 1996; Wills et al., 2002). In comparison, the native chemotype observed in this study, although more similar to the Rg1 and Re compositions reported for wild populations by Assinewe et al. (2003), was novel because levels of Re were much lower than previously reported. It appears, therefore, that native Maryland plants exhibit a chemotype that is different from the chemotypes reported previously for American ginseng roots.

Table 18. Mean ginsenoside composition of cultivated and wild American ginseng populations in Maryland (this study) and in four previous studies.

Production	Reference	Ginsenoside Composition				
		Rg1	Re	Rb1	Rc	Rd
Cultivated	Court et al., 1996b	4	35	45	6	10
Cultivated	Li et al., 1996	6	37	41	6	10
Cultivated	Wills et al., 2002	8	40	45	3	4
Cultivated	Assinewe et al., 2003	5	36	39	7	10
Cultivated	this study, exotic ^a	8	51	27	9	6
Wild	this study, (P5 and P7)	9	45	34	9	3
Cultivated	this study, native ^b	47	9	33	8	3
Wild	this study (P1, P3, P9)	45	11	30	10	4
Wild	Assinewe et al., 2003	16	24	48	7	5
Wild	this study	25	27	35	10	3

^a Exotic = F1MD, F2EX.

^b Native = F2MD, F3MD, F4MD.

When all wild samples were averaged together, regardless of chemotype, Rg1 and Re compositions in this study were more similar to those of Assinewe et al. (2003) (Table 18). However, this was the result of mixing chemotypes and only a few roots in populations F3MD and P5WD actually exhibited intermediate chemotypes similar to those reported by Assinewe et al. (2003). These results suggest that intermediate chemotypes may be the result of hybridization among individuals expressing the two distinct chemotypes observed in this study; however, local adaptation cannot be ruled out and hybridization is clearly speculative. Still, if this assumption was correct, it would further suggest that widespread hybridization

had not yet occurred in all Maryland American ginseng populations. The data from this study suggest that P7WD is an artificially introduced population that is both chemically and genetically distinct from native Maryland American ginseng. Data to support the possibility of exotic introductions into P5WD is less clear. Roots in population P5WD were both phytochemically and genetically unique. However, all plants sampled from P5WD exhibited high levels of Re ginsenoside. Thus, a small number of exotic plants may have been introduced into population P5WD or gene flow from wild exotic populations (such as P7WD) and hybridization may have occurred. In contrast, many of the roots in this study appear to be native and the majority of roots cultivated from seed allegedly collected from wild populations in Maryland do appear to be native. These data suggest that artificial introductions may have occurred in some populations, but many populations, both wild and cultivated, show little evidence of introductions. However, given the prevalence of introductions, there is a high risk of widespread contamination and a high risk that the native chemotype observed in Maryland American ginseng may be lost.

CONCLUSIONS

In conclusion, plants in wild populations in Garrett County and plants grown from seed collected locally from wild populations in Maryland are both phytochemically and genetically distinct from exotic American ginseng plants; however, the persistence of these native plants may be threatened by over-harvest and artificial introductions. Depending on seed source, artificial introductions could hasten the mixing of native and exotic plants and threaten local or native genetic and chemical diversity. In addition, by distinguishing between production type (wild,

cultivated, wild-simulated), but failing to distinguish between plants cultivated from exotic seed versus plants cultivated from native seed, we fail to realize the full benefits of wild-simulated cultivation. Therefore, the creation of a native seed bank is essential to reduce farmers and collectors dependence on exotic seed thereby reducing the threat of widespread contamination. The chemotypic data provided in this study proved to be a good indicator of seed source and diversity. This suggests that chemical profile should be a valuable tool for the efficient selection of diverse native seed accessions.

Appendices

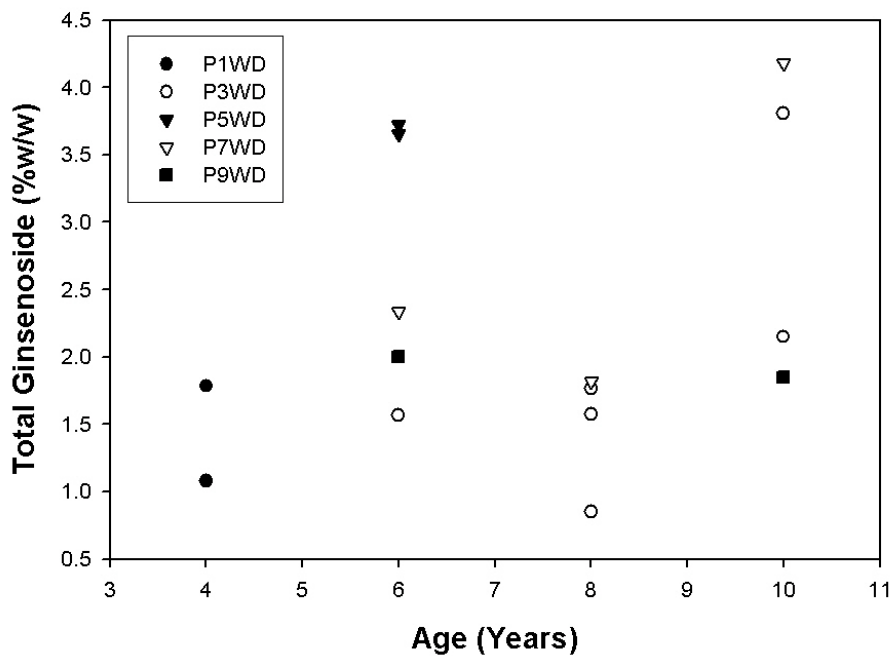
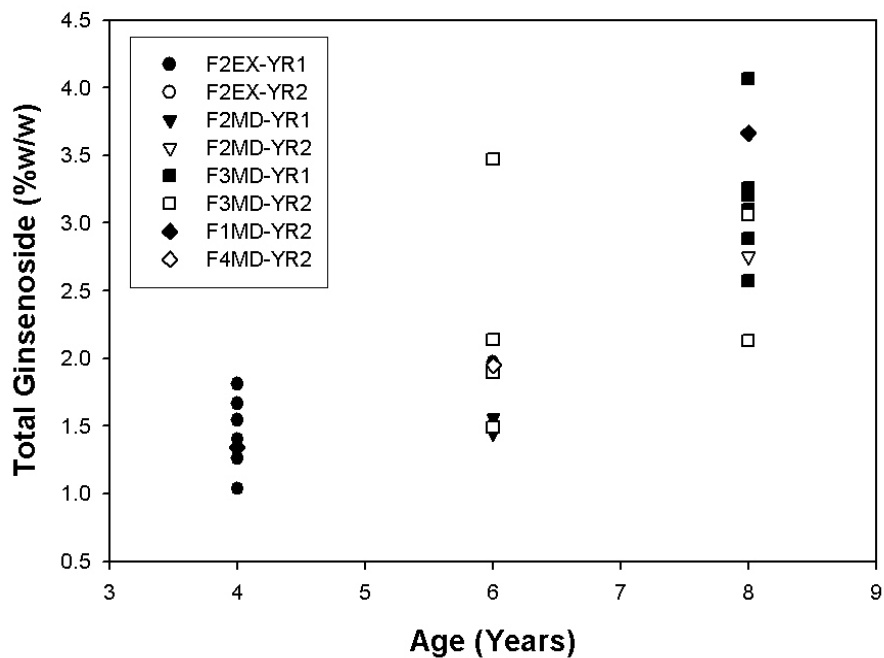
Appendix A. Estimates of population diversity for cultivated populations of American ginseng grown in five counties in Maryland. Standard deviations of the mean genetic distance are given in parentheses.

Population	County	Sample Size	Mean SM ^b
Native seed			
R1	Allegheny	13	0.25 (0.08)
L1	Allegheny	5	0.18 (0.06)
H1	Garrett	17	0.19 (0.06)
P1	Frederick	7	0.27 (0.10)
F1	Washington	2	0.41 (0.00)
G1	Allegheny	3	0.31 (0.04)
P2	Washington	1	n/a
T1	PG ^a	2	0.50 (0.00)
Exotic seed			
L2	Allegheny	12	0.37 (0.09)
G2	Allegheny	3	0.51 (0.06)
F2	Washington	2	0.50 (0.00)

^a PG = Prince George's County.

^b Mean simple-matching genetic distance.

Appendix B. Scatter plots of total root ginsenoside by age class measured in cultivated (top) and wild (bottom) American ginseng grown in Maryland. Cultivated roots are designated by population and year collected. Wild roots are designated by population only. The majority of wild roots were collected the second year (2003).



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