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Genetic Diversity and Variation of Saponin Contents in *Panax notoginseng* Roots from a Single Farm

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Radix notoginseng, the root of *Panax notoginseng* (Burk.) F. H. Chen, has been widely used in traditional Chinese medicine. Its main components, saponins, have been reported to have many pharmacological activities. To test the general assumption that herbs of a single species planted and harvested from a single location are uniform in chemical and genetic makeup, chemical analysis and DNA fingerprinting were carried out. High-performance TLC together with HPLC analysis were used to analyze 17 randomly sampled 3-year-old roots from a single farm for the presence of six saponins. Five roots showed distinct chemical profiles with changed ratios of ginsenosides Rd/Rg1, Re/Rg1, or Rb1/Rg1. The same samples, together with some 1- and 2-year-old samples, were also subjected to fluorescent amplified fragment length polymorphism (AFLP) analysis, and their internal transcribed spacer 2 (ITS 2) regions were sequenced. Fluorescent AFLP analysis was found to be much more polymorphic than the ITS 2 sequence and showed clear evidence of genetic diversity within the tested population. In conclusion, genetic diversity and variation of saponin contents between individual *P. notoginseng* roots have been detected. We suggest that genetic diversity affects the contents of the six saponins. The saponin contents variation and genetic diversity were also found among *P. notoginseng* root samples collected from China and Singapore markets. Since variable saponin contents may affect therapeutic efficacy, combining the use of genetic profiling with chemical profiling will help ensure greater uniformity in the quality of *P. notoginseng* roots. The genetic and chemical diversity within a population also provides the opportunity for breeding new cultivars with more desirable chemical constituents.

KEYWORDS: ***Panax notoginseng*; chemical profiling; ginsenosides; notoginsenosides; AFLP; HPLC; HPTLC; ITS; genetic profiling**

INTRODUCTION

Panax notoginseng (Burk.) F. H. Chen, also known as sanqi or tianqi, is an important herb used in traditional Chinese medicine for promoting blood circulation, removal of blood stasis, induction of blood clotting, relief of swelling, and alleviation of pain (*1*, *2*). *P. notoginseng* is cultivated mainly in Wenshan Prefecture, Yunnan province, China, with its output accounting for 85% of the total production in China (*3*). The chemical constituents in *P. notoginseng* roots include saponins, amino acids, polysaccharides, and flavonoids. Over 20 different saponins, including ginsenosides, notoginsenosides, and gypenosides, are the major bioactive constituents making up 9.75−14.9% of the dry weight. Ginsenosides Rg1, Rb1, and Rd and notoginsenoside R1 are considered to be the main components (**Figure 1**). Research has associated the different saponins to different pharmacological activities. Ginsenoside Rb1 has been shown to prevent memory loss in rats caused by a cholinergic agent (*4*). It was also found to have an antiulcer constituent (*5*). Purified Rb1 sensitized an experimental tumor to ionizing radiation (*6*). Recently, Rb1 was found to have estrogen-like activity (*7*) in activating both α- and β-estrogen receptors in a dose-dependent manner. Rg1, like Rb1, improved spatial learning, increased hippocampal synaptophysin levels in mice (*8*), and also had estrogen-like activity (*9*). Rb1 and Rg1 are, however, opposing active principles in angiogenesis. Rg1 promoted functional neovascularization into a polymer scaffold *in vivo*, while Rb1 inhibited the earliest step in angiogenesis, the chemoinvasion of endothelial cells (*10*). Notoginsenoside R1 was found to increase the fibrinolytic potential in cultured human smooth muscle cells *in vitro*, suggesting its contribution in the treatment of cardiovascular diseases (*11*). Ginsenoside Rd was found to have significant inhibitory activity on both CYP2C9 and CYP3A4 in human liver microsomes, suggesting

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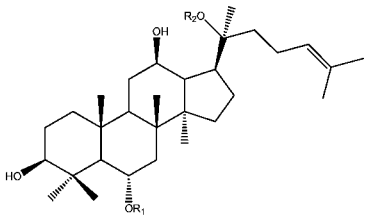
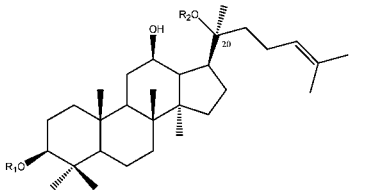


	Saponins	R_1	R_2
1	R1	-Glc^2-Xyl	-Glc
2	Rg1	-Glc	-Glc
3	Re	-Glc^2-Rha	-Glc



	Saponins	R_1	R_2
4	Rb1	-Glc-Glc	-Glc-Glc
5	Rc	-Glc-Glc	-Glc-Ara(f)
6	Rd	-Glc-Glc	-Glc

Figure 1. Chemical structures of six saponins: **1**, notoginsenoside R1; **2**, ginsenoside Rg1; **3**, ginsenoside Re; **4**, ginsenoside Rb1; **5**, ginsenoside Rc; **6**, ginsenoside Rd.

its potential to interact with conventional medicines that are metabolized by CYP2C9 and CYP3A4 *in vivo* (*12*).

DNA fingerprinting or genetic profiling reveals differences in plant genomic DNAs. Various techniques, such as PCR-based random amplified polymorphic DNA (RAPD) (*13*), restriction fragment length polymorphism (RFLP) (*14*), or sequencing of particular genes such as 18S rDNA, chloroplast trnK (*15*), and ribosomal ITS regions (*14*), have been employed to identify *Panax* species and differentiate them from the adulterants. All these works, however, analyzed either a single sample from a population or bulk samples as mixtures of many individuals with the assumption that individuals within the species or populations are genetically and chemically uniform. There has been little attempt to analyze variation in active constituents and genetic makeup within a population.

The objective of this study was to examine uniformity in genetic makeup and chemical composition among individual *P. notoginseng* roots from a single farm. Seventeen individual roots of 3-year-old *P. notoginseng* from a single farm in Wenshan County together with some samples from shops in China and Singapore were analyzed. HPTLC and HPLC chromatographic fingerprints of each sample were obtained, using six saponins (Rb1, Rg1, Rd, R1, Re, and Rc) as marker compounds. Two genetic profiling techniques were employed: DNA sequencing of the ITS 2 region and fluorescent AFLP (*16*). Our multiple technique approach revealed diversity in both saponin contents as well as genetic makeup among the individuals tested. The implications for quality control, GAP farming, and breeding are discussed.

MATERIALS AND METHODS

Materials. Fresh 3-year-old *P. notoginseng* roots were collected from a single farm in October 2002 and sun dried. Randomly chosen samples graded as 30 tou (about 30 roots for every 500 g) were submitted to the laboratory and stored in a dry cabinet before analysis. Five 1-year-old and two 2-year-old plants in the same farm were randomly collected from the same shaded plot and kept under 20 °C before DNA isolation. An authenticated *P. notoginseng* root sample as well as Notoginsenoside R1 standard compound were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) from Beijing, China. Eight other *P. notoginseng* root samples were purchased from various herbal shops in Chengdu and Beijing in China and Singapore. Ginsenosides Rb1, Rc, Rd, Re, and Rg1 standards were purchased from Indofine Chemical Co. (Somerville, NJ).

HPTLC Analysis. About 0.5 g from each of the sun dried *P. notoginseng* root was powdered with a food blender, passed through a sieve, and weighed. Powder was extracted with methanol (1 mL/100 mg) with ultrasound sonication for 30 min in a Branson 8210 sonicator (Danbury, CT). Extract was passed through a 0.45 μm nylon filter and dried under vacuum. Dried extract was redissolved in methanol (1 mL for extract from 0.5 g of powder). HPTLC plate silica gel 60 F_{254} 20 $\times$ 10 cm from Merck (Germany) was used as the stationary phase. Three microliters of sample was sprayed on the silica gel plate as 6 mm bands (2 mm apart) by nitrogen gas with a Linomat 5 (CAMAG), 15 mm from lower edge of the plate. Development was conducted in a 20 $\times$ 10 cm CAMAG twin trough chamber, saturated for 20 min, with 10 mL of developing solvent (chloroform:methanol:water = 13:7:2) per trough at a developing distance of 90 mm from the lower edge of the plate. The plate was dried in a fumehood, sprayed with 10% H_2SO_4 in methanol, dried by heating at 100 °C for 10 min on a CAMAG plate heater before visualization, and photographed under visual or UV light (366 nm) in a CAMAG Reprostar 3 chamber. Loading volumes were adjusted later to give Rg1 a uniform intensity (**Figure 2**).

HPLC analysis. Each *P. notoginseng* root was first washed with 70% ethanol for 1 min to disinfect its surface and remove contaminants. After rinsing twice with sterile water, the roots were dabbed dry and air-dried. 1 g of root was extracted for HPLC analysis as previously reported (*17*). Each individual root was extracted three times. Dried extract was redissolved in 70% methanol before HPLC analysis. An Agilent 1100 liquid chromatograph (Palo Alto, CA) equipped with quaternary gradient pump, degasser, autosampler and photodiode array UV detector was used. HPLC was carried out using a reversed-phase column (Waters Symmetry $C_{18,}$ 250 $\times$ 4.6 mm i.d., 5 μm). The binary gradient elution system consisted of water (A) and acetonitrile (B). Separation was achieved using the following gradient: 0−30 min, 20% B; 30−60 min, 20−45% B; 60−78 min, 45−75% B; 78−80 min, 75−100% B. The column temperature was maintained at 35 °C. The flow rate was 1 mL/min and the injection volume was 5 μL. The UV detection wavelength was set at 203 nm and diode array scanning was from 190 to 400 nm.

Isolation of genomic DNAs. DNA extraction was carried out using a modified CTAB method (*18*). Roughly 1 g of material was cut out from each cleaned root and then ground in a mortar with pestle in the presence of nitrogen. Then 5 mL of extraction buffer [0.35 M glucose, 0.1 M Tris-HCl (pH 8.0), 5 mM EDTA, 2% PVP-40, 1% β-mercaptoethanol] was added and the mixture centrifuged at 5000*g*, 4 °C, for 20 min. The pellet was resuspended in 5 mL of nuclei lysis buffer [1.4 M NaCl, 0.1 M Tris-HCl (pH 8.0), 20 mM EDTA, 2% cetyltrimethylammonium bromide (CTAB), 2% PVP-40, 1% β-mercaptoethanol] and incubated at 65 °C for 1 h. An equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added and the resulting emulsion centrifuged at 5000*g*, 4 °C, for 15 min. The aqueous phase was transferred to a new tube and a 0.6$\times$ volume of 2-propanol added. The genomic DNA was pelleted by centrifugation at 5000*g*, 4 °C, for 30 min, washed with 70% ethanol, and resuspended in $TE_{0.1}$ buffer (20 mM Tris-HCl, 0.1 mM EDTA), and their quality was verified by 1% agarose gel electrophoresis. One representative sample of *Panax quinquefolium* from our previous work (*19*) was included in both ITS 2 region analysis and AFLP analysis as the control.

Amplification and Analysis of ITS 2 Sequences. One primer located in the 5.8S rDNA (5.8d: 5′ AAC CAT CGA GTC TTT GAA CGC A 3′) and another primer located in 28S rDNA (28cc: 5′ ACT CGC CGT TAC TAG GGG AA 3′) were used to amplify the ITS 2 region. PCR in a MJ PTC-100 thermocycler (Watertown, MA) was carried out in a 50 μL reaction mixture containing 1$\times$ Qiagen Taq buffer (Germany), 0.2 mM dNTP, 5 pmol of each primer, 10−50 ng of template DNA, and 1.5 units of Taq polymerase (Qiagen). Samples were denatured at 90 °C for 3 min and subjected to 35 cycles of 1 min at 90 °C, 1 min at 55 °C, 1.5 min at 72 °C, and a final extension step

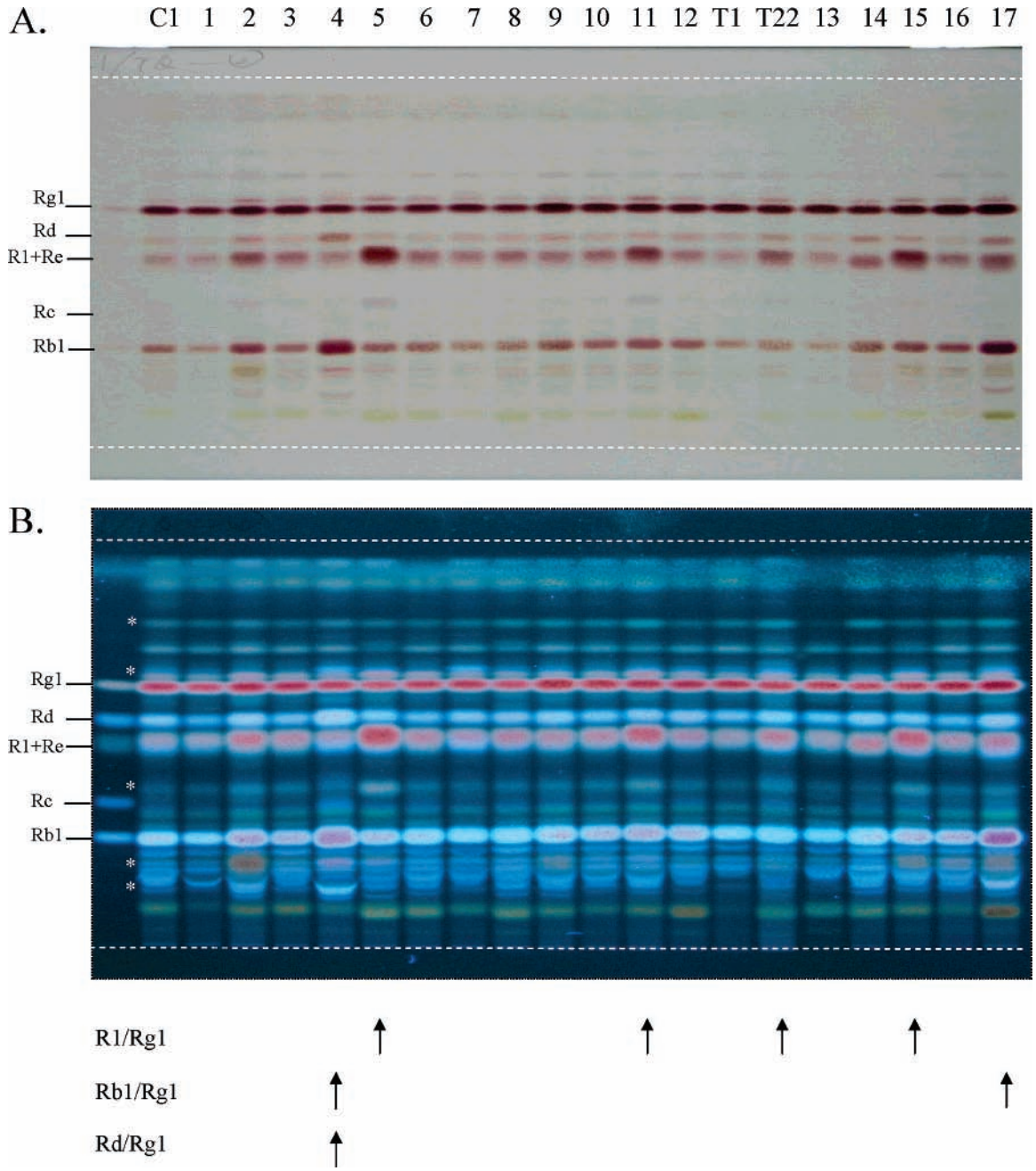


Figure 2. HPTLC chromatograms of extracts from *P. notoginseng* roots. C1, the authenticated sample from NICPBP; 1–17, individual roots from the farm; T1, a sample collected from one herbal shop in Chengdu, China; T22, a sample collected from a herbal shop in Singapore. (**A**) Photography under daylight after derivatization with H_2SO_4 in methanol and heating at 100 °C for 10 min. (**B**) Photography of the same plate under 366-nm irradiation after derivatization. Saponin standards: Rg1, 0.125 μg; Rb1, 0.59 μg; Rc, 0.16 μg; Rd, 0.12 μg; Re, 0.2 μg; R1, 0.4 μg.

of 10 min at 72 °C. Two microliter of the derived cDNA inserts were used for fluorescent cycle sequencing with Applied Biosystem BigDye terminator sequencing reagent in a volume of 20 μL using 5.8d or 28cc as primer (96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min, 30 cycles). Sequences were determined by an Applied Biosystem 3730xl DNA analyzer. Sequence analysis was carried out using the MegAlign program by DNASTAR (Madison, WI). An ITS 2 sequence for *Panax ginseng* from Genbank (accession no. AB043872) was also included in the sequence alignment.

Fluorescent AFLP Analysis. DNA digestion (~0.5 μg) by *EcoR* I and *Mse* I, ligation to adaptor, preamplification, and amplification steps were all performed according to the protocol of an AFLP Plant Mapping Kit from Applied Biosystem. For each sample, two specific amplifications were performed with *EcoR* I-ACT/*Mse* I-CTG and *EcoR* I-ACG/*Mse* I-CAA as previously reported (*19*). The *EcoR* I primers were labeled with the fluorescent dye FAM (blue) at the 5′ end. After mixing with DNA size standard (50−500 bp) labeled with ROX (red), amplified fragments were separated on polyacrylamide gel using an ABI 377 DNA sequencer. Data of fragments were processed with a Genescan genotyper (Applied Biosystem) for sizing in reference to internal size standards. Clear and distinct bands were scored as 1 (present), and the similarity index between test samples was calculated according to a simple matching method with the formula SI = (n11 + n00)/*n* where n11 is the number of bands shared between two samples, n00 the number of bands absent from both samples, and *n* is the total number of bands. The UPGMA algorithm was used for clustering and tree drawing with the software package NTSYSpc by Exeter Software (New York, NY).

RESULTS AND DISCUSSIONS

Notoginsenoside R1 and ginsenosides Rg1, Rb1, and Rd are the main active constituents in the root of *P. notoginseng*,

accounting for about 80% of total saponins. They are different from each other according to their carbohydrate side chains at three positions (**Figure 1**). When the extracts from individual *P. notoginseng* roots were subjected to HPTLC analysis, the four saponins were well separated as the main bands detected (**Figure 2**). More sensitive results with more than 15 well-separated bands for each sample were obtained when checking the plate under 366 nm UV light after H_2SO_4 spraying (**Figure 2B**). Despite the fact that R1 overlapped with another ginsenoside (Re), R1 was the main component for the band, as revealed by our follow-up HPLC analysis (**Figure 3**). With this HPTLC system, *P. notoginseng* root samples were distinct from those of related species of *P. ginseng* and *P. quinquefolium*. Raw and steamed *P. notoginseng* products could also be clearly differentiated (data now shown). Serial titration of Rg1 and Rb1 indicated that the visually observed intensity correlated positively with loading quantity. Detection limits (80−200 ng) for this HPTLC system for the six saponins were comparable with HPLC analysis. With good separation of the major saponins and high sensitivity, HPTLC is suitable for profiling saponin contents in *P. notoginseng* roots. Other advantages include good throughput (as many as 50 samples can be analyzed by one technician daily), ease of scaling up, lower setting up cost, and lower operation cost. The colorful chromatograms are easy to comprehend and compare. These are all important factors for large-scale analysis, such as the screening of thousands of roots for breeding purpose. The use of a suitable scanner and calibration curve makes quantitation of saponins possible.

All the *P. notoginseng* root samples shared a distinct chemical profile in which four major bands corresponded to the four major saponins Rg1, Rb1, R1, and Rd. We focused on the relative abundance of the four saponins instead of the total saponin content. Most samples analyzed shared the same pattern as the authenticated sample, with the exception of a few. Out of the 17 3-year-old roots, sample 4 was very different in that there was higher abundance of Rd and Rb1 relative to Rg1. A higher abundance of Rb1 was also observed in sample 17. Sample 5, on the other hand, had higher abundance of R1 + Re than the standard sample. Such higher abundance of R1 + Re was also observed in samples 11 and 15 and a *P. notoginseng* root purchased from Singapore (T22 in **Figure 2**). Besides the differences for the four major saponins, other minor differences were also observed for ginsenoside Rc and other bands, as indicated by asterisks (**Figure 2B**). These bands may be of other saponins or other chemical components. It is well-accepted that soil conditions, weather conditions, and agricultural practices like fertilization, harvest time, and processing would all affect the chemical composition of medicinal herbs. Our choice of random samples of 3-year-old roots of similar size (the same grade) from one batch of plants from a single farm that were grown, harvested, and processed in the same way eliminated most, if not all, variations of external factors that might affect the relative abundance of the major four saponins. This result strongly suggests that the differences were determined genetically.

To confirm the variation of abundance of saponins, quantitative analysis by HPLC was carried out on 12 of the 17 samples. The six saponins were well-separated (*17*) by HPLC analysis (**Figure 3**). The peaks were identified by retention time and spiking of known standards. Using the established HPLC method (*17*), the amounts of six saponins were calculated from the calibration curves that were done prior to the analysis of the samples. Triplicate extracts of the same sample were analyzed. Their average values are shown in **Table 1** and the RSD of the six saponins in the different extracts range from 1.2 to 9.6% (average of 7.1%). Intra- and interday variation (RSD) of the contents of the six saponins were 0.1−4.8% ($n = 5$) and 1.5−8.7% ($n = 3$), respectively. Recoveries of the saponins were in the range of 97−102% ($n = 3$, RSD < 6.4%).

As indicated in **Figure 2** and **Table 1**, the chromatographic fingerprints of most of the roots were consistent with those previously reported (*20*) as well as that of the authenticated sample. The total contents of the four major saponins (R1, Rg1, Rb1, and Rd) in the different individual roots ranged from 45.6 to 89.1 mg/g, with samples 3 and 4 having the highest total content of saponins, while samples 1 and 2 have the lowest total content. The total contents were generally lower than the previously reported contents (76.7−112.9 mg/g) in the roots obtained from different subregions of Wenshan Prefecture (*3*), although the roots in this study were also obtained from a farm in Wenshan County. In terms of individual saponins, notoginsenoside R1 contents in all the analyzed samples (5.9−36.6 mg/g) were found to be 1.8−15.4 times higher than those from the previous study (*3*).

The R1/Rg1 ratios in samples 5 and 11 and the Rb1/Rg1 and Rd/Rg1 ratios in sample 4 were indeed outliers (analyzed using Boxplots from SPSS) compared to the respective ratios in other samples (**Table 1**). Abundance of Rb1 and Rd relative to Rg1 was higher for sample 4. The Rb1/Rg1 and Rd/Rg1 ratios were 3.61 and 0.80 in sample 4 compared to 0.90 and 0.22 in the authenticated sample (TQC1). For sample 5, R1 relative to Rg1 (3.42) (**Figure 3B** and **Table 1**) was substantially higher than that of the authenticated sample (0.29). Similarly for sample 11, the ratio of R1 to Rg1 was 1.4. These observations from HPLC correlated well with the results from HPTLC analysis. Compared to the previous study (*3*), R1/Rg1 ratios were much higher for all the samples. These results showed that there is a great variation in the relative abundances of the saponins in the roots obtained from the same farm. As saponins are the active constituents of *P. notoginseng*, the wide variations in their contents and relative abundances may affect their pharmacological activities. Therefore, it is important to ensure the consistency of their saponin contents.

Genetic differences, or differences in genomic DNAs, are regarded as a more discrete, standard, and definite means of botanical identification than morphology observation and chemical methods. Various techniques have been successfully employed to identify different *Panax* species and differentiate them from the adulterants (*21*). Techniques such as AFLP and microsatellite DNA provided sufficient resolution to distinguish *P. quinquefolium* samples from different farms (*21*). All the experiments analyzed either a single sample as the representative of a farm, a location, or a species with the underlying assumption that the populations analyzed were uniform or that the difference within a population is negligible. Such assumption has been proven right for vegetatively propagated plant populations (*22*) but remains to be proven in seed-derived populations.

In this study, ITS 2 sequence and fluorescent AFLP analysis were employed to understand the genetic diversity within a population from a single farm. Besides the same 17 samples used for chemical composition analysis, five randomly chosen 1-year-old plants and two randomly chosen 2-year-old plants were also included, together with the *P. notoginseng* roots purchased from various herbal shops in China and Singapore. The noncoding region between 5.8S rDNA and 28S rDNA, termed internal transcribed spacer 2 (ITS 2), has been used widely to identify botanical origin of some herbal drugs as well

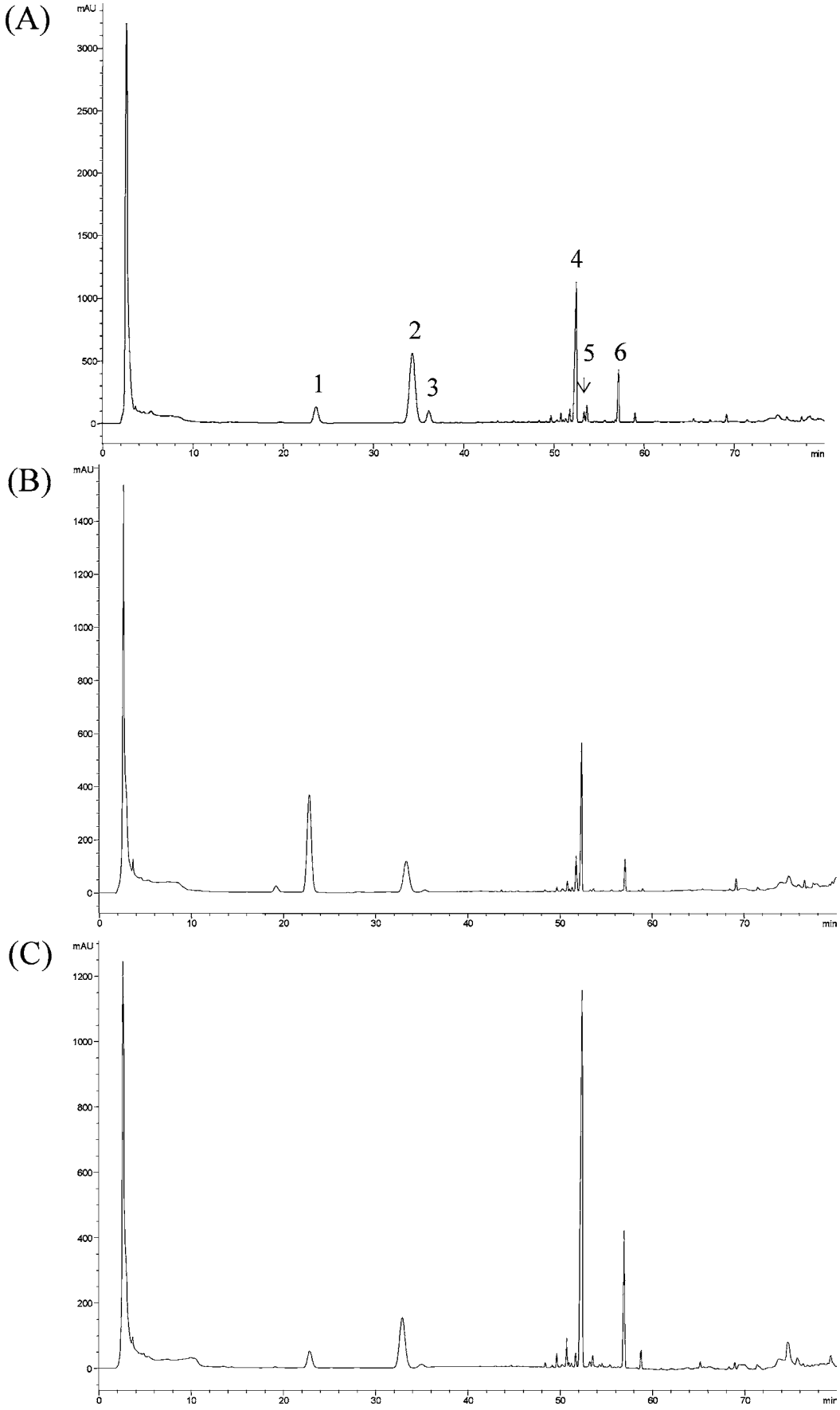


Figure 3. HPLC chromatograms of extracts of (**A**) TQC1, the authenticated *P. notoginseng* sample from NICPBP, and (**B**) sample 4 and (**C**) sample 5 from the farm. Peak 1, notoginsenoside R1; 2, ginsenoside Rg1; 3, ginsenoside Re; 4, ginsenoside Rb1; 5, ginsenoside Rc; 6, ginsenoside Rd.

Table 1. Concentration of Six Saponins (mg/g) in the Different Roots ($n = 3$)[a]

samples	R1	Rg1	Re	Rb1	Rc	Rd	total (all)	sum of four[b]	R1/Rg1	Rb1/Rg1	Rd/Rg1
TQC1	7.36	25.23	4.56	22.76	1.35	5.61	66.87	60.97	0.29	0.90	0.22
1	7.87	22.65	3.10	12.63	0.69	2.45	**49.40**	**45.60**	0.35	0.56	0.11
2	11.10	13.40	2.59	19.03	0.84	2.80	**49.76**	**46.33**	0.83	1.42	0.21
3	19.50	**38.20**	**8.53**	25.69	1.61	5.73	**99.28**	**89.13**	0.51	0.67	0.15
4	6.23	14.83	1.52	**53.51**	1.09	**11.85**	**89.03**	**86.42**	0.42	**3.61**	**0.80**
5	**36.58**	10.69	0.84	15.54	0.13	2.95	66.74	65.76	**3.42**	1.45	0.28
6	12.02	21.29	6.68	29.45	1.67	3.93	75.04	66.69	0.56	1.38	0.18
7	6.88	22.32	3.51	21.97	0.88	4.94	60.49	56.10	0.31	0.98	0.22
8	15.27	22.42	3.10	34.19	**3.44**	4.96	83.38	76.84	0.68	1.52	0.22
9	5.93	30.33	3.35	22.21	0.86	5.10	67.78	63.57	0.20	0.73	0.17
10	13.38	26.23	6.44	17.59	0.91	3.68	68.23	60.88	0.51	0.67	0.14
11	23.27	16.95	3.95	20.45	0.80	4.56	69.98	65.23	**1.37**	1.21	0.27
12	10.10	24.48	1.76	28.05	0.87	5.99	71.24	68.62	0.41	1.15	0.24

[a] The values in bold were analyzed to be outliers using Boxplot (SPSS). [b] R1 + Rg1 + Rb1 + Rd.

as to resolve phylogenetic relationship (*23*). This region has been found to evolve faster than the rDNA regions, thereby providing a better resolution. In our study, the ITS 2 regions of all samples were amplified and directly sequenced. Alignment of the 235 bp ITS 2 regions revealed some distinct differences between *P. notoginseng* samples and those of *P. quinquefolium* and *P. ginseng* at eight locations (**Figure 4A**): 26, 30, 32, 41, 44, 138, 205, and 216 bp. *P. quinquefolium* and *P. ginseng* ITS 2 regions were much closer, with a difference of only two nucleotides. Among the *P. notoginseng* samples, most shared the same ITS 2 sequence as the authenticated sample, with only eight samples showing one or three nucleotide differences at five locations (see **Figure 4A** for details). We concluded that this technique was not sensitive enough to demonstrate polymorphism among the individuals.

AFLP is a more robust technique, the outcome of which does not rely on any single gene. It samples the entire genome by amplifying a subset of fragments generated by restriction digestion of target genomic DNAs (*16*). With the incorporation of a fluorescent dye during amplification and separation of fragments by an automated DNA sequencer followed by fragment analysis based on internal size standards, fluorescent AFLP has been regarded as a very sensitive and reliable technique. Our previous work indicated that partial degradation due to sun drying and normal storage had little impact on AFLP results (*19*). In this study, we also found that the DNAs were of sufficient quality. With the two sets of primer combinations (*19*), 159 discrete polymorphic bands were obtained and included in phylogenetic analysis. As a group, all *P.* notoginseng samples were genetically distinct from the sample of *P. quinquifolium* unambiguously (**Figure 4B**). Among the *P. notoginseng* samples, varied levels of genetic diversity were observed. The 17 3-year-old plants were distributed within the two genetic groups (met at similarity index 0.75), with the authenticated sample belonging to the smaller group. All year 1 and 2 plants belonged to the larger group. The two year 1 plants with most distinct morphological features (Y1-1 had yellowish leaves, while Y1-2 had red pigments in the middle portion of stem) were genetically more distinct from the other three with green leaves and almost no red pigment in the stem. In this aspect, the degree of genetic diversity correlated with morphological differences. The eight *P. notoginseng* root samples were purchased from various herbal shops in China and Singapore, and we presumed that most of them originated from different production areas in China, or at least from different farms. These samples distributed equally in the two groups and were not distinct from those from the farm. It is noted that four of these commercial samples clustered closer to the authenticated sample.

The comparison of saponin contents variation with genetic diversity presented some interesting results. The root with the most difference in saponin contents (Y3-4) was also an outlier in the large group. Similarly, the root with higher abundance of R1 (Y3-15) was also an outlier. However, the two other three-year-old roots (Y3-5 and Y3-11) with higher R1 abundance were not outliers, with closely related samples showing no obvious change in saponin contents. Moreover, some samples with a more distinctive genetic makeup like Y3-13 and T7 did not show much variation in the relative abundance of the saponins tested. We conclude that AFLP analysis clearly indicated genetic diversity among the samples from the farm. Such genetic diversity had contributed to the variation of saponin contents. There is, however, no strict correlation of relative abundance of saponins tested with particular genetic makeup. Several factors may explain such loose linkage. First, most genetic changes occur at noncoding sequences, and these noncoding sequences may comprise the bulk of genomes in plants. Second, many changes in coding regions are silent and do not lead to amino acid change. Third, only a handful of proteins are involved in saponin biosynthesis among numerous proteins, and changes in most proteins may have no impact on saponin biosynthesis. Linkage of a particular band or pattern to a desirable saponin content is only possible with both genetic and chemical analysis of a much bigger population followed by statistical analysis.

The genetic and chemical composition diversity found in our study highlighted the lack of uniformity in genetic makeup and contents of the six saponins in a *P. notoginseng* farm in Wenshan. Morphological variation within the farm could be observed from 1-year-old plants onward. When judged by the criteria set up by the International Union for the Protection of New Plant Varieties (UPOV), the plants analyzed in our study could hardly be regarded as one variety. There is concern that products from such farms can vary in their saponin contents and therefore in clinical efficacies.

Chemical assessment of roots of *P. notoginseng* by systematic analysis of plants from different parts of China and different areas of Wenshan Prefecture was reported (*3*). The impact of different seasons of harvest and market grades on quality was also evaluated. Using total saponin content as the criteria for assessment, the authors concluded that the Southwestern region of Wenshan Prefecture including Wenshan County produced superior *P. notoginseng* roots and suggested geographic location as the cause of difference. Our finding puts a question mark on

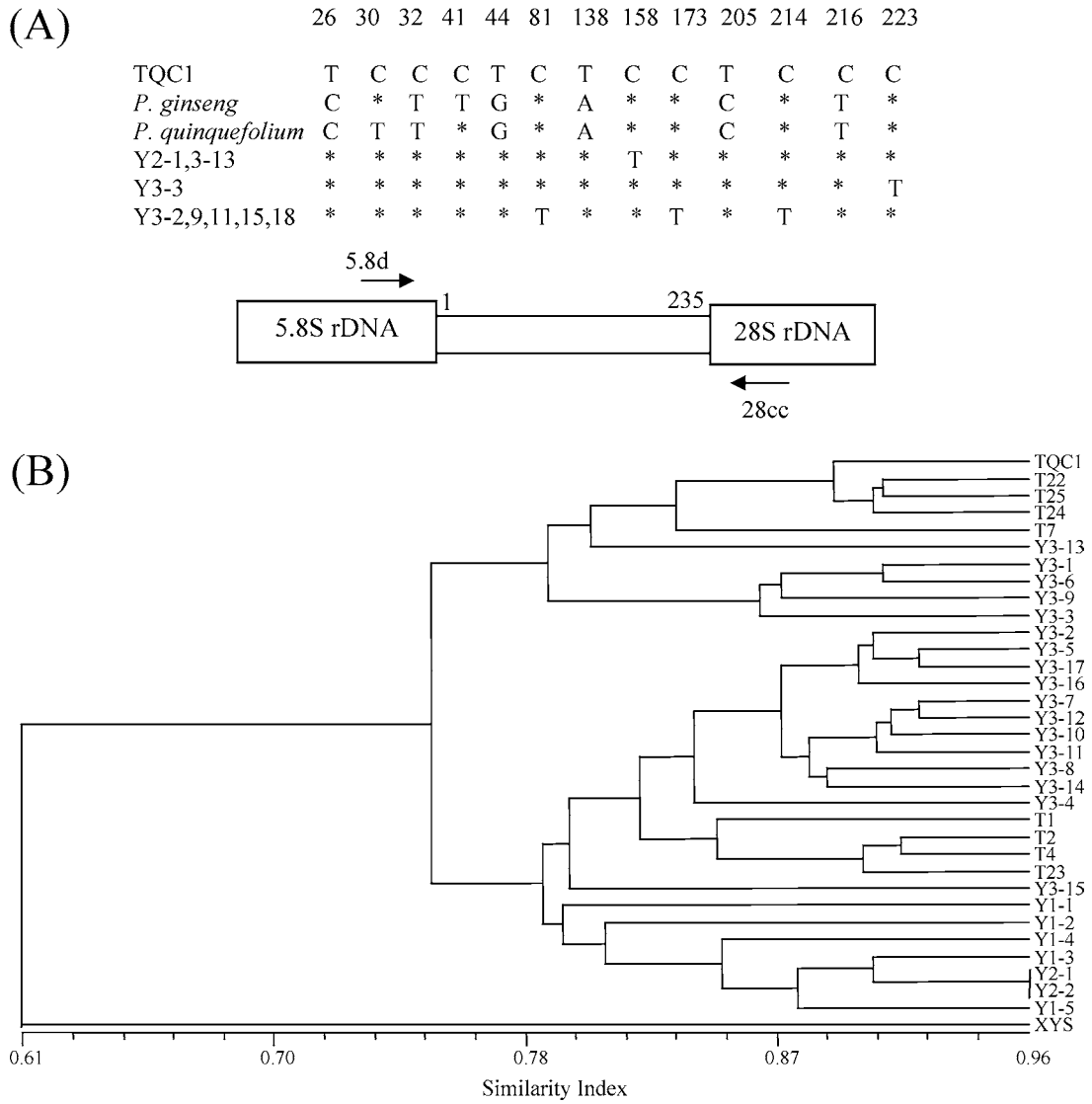


Figure 4. Genetic relatedness of samples tested as revealed by ITS 2 region alignment and AFLP analysis. (**A**). Sequence comparison of the ITS 2 regions. The numbers above sequences are the aligned nucleotide positions. Asterisks indicate the nucleotides identical to the authenticated sample (TQC1). (**B**). Phylogenetic relationship of the 3-year-old *P. notoginseng* plants (Y3-1 to Y3-17), year 1 and year 2 plants (Y1-1 to Y1-5, Y2-1, and Y2-2) from the same farm in comparison with the authenticated sample (TQC1), a *P. quinquefolium* sample (XYS), and other samples purchased from different herbal shops (T1, T2, T4, T7, T22, T23, T24).

such a conclusion based solely on chemical analysis, since contribution by better varieties cannot be excluded. The relative contribution by genetic and geographic factor remains to be investigated. If the genetic makeup of a plant is a major contributor, then other regions regarded as "poor producers" can improve by switching to plants of better quality. Any strong conclusion, however, will only be reached after systematic analysis of saponin contents in plants of genetically uniform cultivars planted in multiple geographic locations and plants of genetically distinct cultivars planted in one location.

While there have been intensive breeding activities for crops, there has been little attention paid to the breeding of medicinal herbs. The many reasons include difficulties in evaluating quality of medical herbs, lack of direction for breeding, and the absence of a cost-effective technique to check for breeding objective. *P. notoginseng* has been grown in Wenshan for over 1000 years. The breeding efforts were limited to the production of bigger plants with heavier roots. With more pharmacological properties attributed to individual saponins, there are now some clear targets for breeding *P. notoginseng*. Since Rd is potentially involved in herb−drug interactions, cultivars with less or little Rd will be very desirable and beneficial. Cultivars with a higher relative ratio of R1 could be more effective in treating cardiovascular diseases. Rb1 and Rg1 are opposing active principles in angiogenesis, more Rg1 would be needed for wound healing while the inhibitory activity of Rb1 will be useful for cancer treatment. Our study also demonstrates that HPTLC could be effectively used in chemical component directed breeding because of its high throughput, low operation cost, and strong correlation with HPLC in evaluation of relative saponin abundance. More importantly, the diversity of chemical composition and genetic makeup within a single farm exists as a ready and valuable source for breeding. More genetic analysis can be conducted, together with chemical analysis and bioassays, and genetic markers can be identified. These markers with strong linkage to breeding target could be applied to assist breeding and shorten the breeding cycle.

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