Genetic Diversity and Variety Identification of *Panax* Species Based on EST-SSR Markers

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Research Article

Keywords: EST, Panax ginseng, Panax quinquefolium, Panax notoginseng, SSR

Posted Date: August 17th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-800169/v1

Abstract

Background: The roots of *Panax* species are widely used in the East because of their high medicinal and economic value. They are similar in plant morphology and chemical composition, but have quite differences in medicinal properties and efficacy, therefore, genetic diversity and variety identification of *Panax* species is particularly important.

Methods: We screened 7 Simple Sequence Repeat (SSR) markers from expressed sequence tags (ESTs) database of *Panax* species in NCBI. Using these markers test SSR polymorphism in *Panax* species.

Results: Seven SSR markers could successfully identify *Panax ginseng, Panax quinquefolium, Panax notoginseng,* and their commercial products. Among three ginseng varieties, garden ginseng, forest ginseng, and wild ginseng, the polymorphism of EST-SSR markers decreased gradually, which may be related to age and environment. Two pairs of EST-SSR primers can specifically identify three ginseng cultivars. The phylogenetic relationships analysis showed that *Panax ginseng* and *Panax quinquefolium* were closer than *Panax notoginseng*. Compared with wild ginseng, the relationship between the garden ginseng and the forest ginseng was closer.

Conclusion: SSR molecular markers have high repeatability and can be used as reliable molecular markers for genetic diversity and variety identification of *Panax* species.

1. Introduction

Panax ginseng, Panax quinquefolium, and *Panax notoginseng* are precious traditional Chinese herbal medicines, which have been widely used in clinical treatment for hundreds of years. *P. ginseng* has the functions of regulating the central nervous system, lowering blood sugar, enhancing human immunity and adaptability, and resisting the spread and metastasis of cancer cells (Mancuso et al. 2017). Besides lowering blood pressure, *P. quinquefolium* also has the functions of reducing blood lipids, improving myocardial ischemia, and enhancing central nervous system (Chen et al. 2008). However, *P. notoginseng* has anti-inflammation, anti-oxidation, inhibition of platelet aggregation, regulation of blood glucose and blood pressure, inhibition of neuronal apoptosis, and neuronal protection (Zhang et al. 2008). The three *Panax* species have certain similarities in plant morphology and chemical composition, but they have great differences in medicinal properties and efficacy. So they cannot be mixed.

According to the different growth environments and cultivation methods of *P. ginseng*, the Chinese Pharmacopoeia divides cultivated ginseng into two types. One is ginseng grown in gardens, commonly known as garden ginseng, and the other is artificially planted ginseng that grows naturally in mountain forests, often called mountain-cultivated ginseng or forest ginseng (Liu et al. 2008; Pan et al. 2013). Besides cultivated ginseng, the original wild ginseng growing naturally in the deep mountains is the best quality ginseng, and its quantity is extremely rare (Li et al. 2017). According to the Chinese wild ginseng grading quality standards, ginseng that has grown naturally in mountain forests for more than 15 years is called wild ginseng. As we all know, three subspecies of *P. ginseng*, garden ginseng, forest ginseng, and

wild ginseng, have different chemical compositions and bioactive substances due to the diverse growth environments and ages (Zhu et al. 2018). However, the differences in medicinal value and economic value lead to the phenomenon that garden ginseng is used as forest ginseng and forest ginseng is used as wild ginseng (Zhang et al. 2020). Therefore, it is essential to develop an effective method to identify three varieties for maintaining public health as well as for protecting the rights of consumers.

In the past few decades, morphological and chemical analysis methods have been used as the main identification methods of *Panax* species. However, morphological methods require long-term experience, which is greatly affected by subjective factors. Furthermore, it is impossible to use morphology to identify the raw materials of commercial products, because these products usually are sold in the form of powder, pellets, extract, dried roots, shredded slices, and tea powder (Jung et al. 2014). The chemical analysis method, which focuses on the analysis of ginsenosides, has disadvantages such as expensive equipment, long detection time, and cumbersome sample pretreatment procedures, etc. With the development of molecular biology, the application of DNA molecular marker technology has made the identification of Chinese herbal medicines more accurate and convenient (GarridoCardenas et al. 2018). Compared with other identification methods, DNA molecular markers have the advantages of less sample consumption, short detection period, high polymorphism, stable and reproducible results (Good et al. 2019).

Recently, various DNA molecular marker systems - including Random Amplified Polymorphism DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), and Single Nucleotide Polymorphism (SNP) - have been developed for identifying *P. ginseng, P. quinquefolium* and *P. notoginseng* (Jung et al. 2014; Ngan et al. 1999; Um et al. 2001; Shaw et al. 1995; Choi et al. 2008; Liu et al. 2006). However, there are few studies on the differences in polymorphism among three subspecies of *P. ginseng*, garden ginseng, forest ginseng, and wild ginseng. Compared with other DNA molecular markers, SSR markers are characterized by abundant quantity, good repeatability, uniform distribution, and high polymorphism, which is beneficial for the identification of plant germplasm resources, analysis of genetic diversity, and construction of DNA fingerprint map (Jang et al. 2020). However, due to the lack of complete genome and transcriptome sequence information of *Panax* species, it is difficult to get sufficient DNA molecular markers. The purpose of this study is to develop SSR markers based on expressed sequence tags (ESTs) of *Panax* species, and apply them in the identification of fresh samples and commercial products, which will provide a new method for the identification of *Panax* species and their commercial products in the future.

2. Materials And Methods

2.1 Plant materials and DNA extraction

Samples of *P. quinquefolium* and *P. notoginseng* were provided by Jilin Yisheng Hansen Biotechnology Co., Ltd. The garden ginseng, forest ginseng, and wild ginseng were provided by the National Ginseng Products Quality Supervision Inspection Center. Various commercial products were purchased from

ginseng market, including powder, dried roots, and shredded slices. The modified cetyltrimethylammonium bromide method was used to extract DNA.

2.2 Screening of SSR primers

All ESTs of *Panax* species were downloaded from NCBI database. The SSR locis were searched by SSRIT (http://www.gramene.org/db/searches/ssrtool) after the sequence pretreatment with EGassembler (http://www.genome.jp/tools/egassenbler/). The primers on flanking sequences on both sides of SSR motif were designed with Primer3(http://primer.ut.ee/). The principles of primer design were as follows: Tm 55–60 ℃, GC content 40–60 %, primer length 18–23 bp and product size 100–400 bp (Table.1).

2.3 Polymerase chain reaction amplification

Polymerase chain reaction (PCR) was performed in 25 μL reaction volumes containing the following: 40 ng genomic DNA, 10 pmol each primer, and 12.5 μL premix rTaq (TaKaRa, Dalian, China). Amplification was performed as follows: 5 min at 94 ℃ for initial DNA denaturation; 30 cycles of 94 ℃ 30 s, 46 or 55 ℃ 30 s, and 30 s at 72 ℃; followed by 10 min at 72 ℃. By screening 40 polymorphic SSRs, we tried to select a set of SSR polymorphic markers showing clear and reproducible band profiles that could be used to discriminate *Panax* species (Table 1). SSR polymorphism was detected by non-denaturing polyacrylamide gel electrophoresis (native-PAGE) and capillary electrophoresis. For phylogenetic analysis, we used 27 alleles of seven polymorphic markers. Phylogenetic analysis among *Panax* species was conducted using the unweighted pair group method with arithmetic means clustering in the NT-SYSpc ver.2.1 (Exeter Software, New York, NY, USA).

Table 1
EST-SSR primer sequences and fragments resulting from each pair of primers

Primer number	Repetitive motif	Primer sequence (5'-3')	Product size / bp	annealing temperature/℃
P2	(TA)10	F: ACTACCGGAGTCAAGCCTCA R: ACGGCCATCATTAATCCAAA	209	55
P17	(TA)7	F: AGCAAGCTTCTGATTCTGTGG R: ATGCACCTGACTCGTAGGCT	250	55
P35	(GCT)9	F: AAAGCAGGGTGTGCTCACTT R:AGGGAGACCGGAGCATTATT	198	55
Q2	(AT)7	F:TCCAAAGACCCCTCCTCCAA R:TGGTGAGTGGCTGAATCTGG	160	46
Q3	(CT)7	F: GCTGGTCCCCTCTACAACAC R: GCCATCGTTTTGTCCTCGTC	352	46
Q5	(TA)9	F: GCAGGTGTTCACATACTTCGC R: AGGAATCAGAAGAAGGAGCCC	300	46
Q8	(TG)8	F:CCGGGGATTGAAGCAGGAAT R:CACCCGGCCCACACATATAT	208	46
Q11	(TG)8	F:CCGGGGATTGAAGCAGGAAT R:CACCCGGCCCACACATATAT	208	46

3. Results

3.1 Identification of P. ginseng, P. quinquefolium and P. notoginseng using EST-SSR markers

We developed 40 polymorphic EST-SSR markers using bioinformatics methods, among which 15 were polymorphic in *P. ginseng, P. quinquefolium*, and *P. notoginseng*. Considering the factors related to EST-SSR markers such as PIC values, allele copy number, genome distribution uniformity, and PCR amplification efficiency, seven pairs of polymorphic markers - P2, P35, Q2, Q3, Q5, Q8, and Q11 - were selected to specifically identify *P. ginseng, P. quinquefolium* and *P. notoginseng* (Table 2). Each pair of these EST-SSR primers could detect different numbers of alleles (range, 3 to 5; mean 4). Polymorphism

information content (PIC) values ranged between 0.59 and 0.74, with an average of 0.65. Their amplification bands were legible, easy to count, and distinguishable from each other. Two of them had three-nucleotide SSR motifs and the other five had two-nucleotide SSR motifs, of which two were $(TA)_2$ motifs.

Table.2. Marker combinations for each *Panax* species

Alleles of each primers	No of alleles	Polymorphism information content	Cultivar name		
			Panax ginseng	*Panax quinquefolium*	*Panax notoginseng*
P2	5	0.672	b,d	c	a,e
P35	3	0.59	a	c	b
Q2	5	0.74	d	a,e	b,c
Q3	3	0.59	b	a	c
Q5	5	0.74	c,e	b,d	a
Q8	3	0.55	c	a	b
Q11	4	0.7	b	c	a,d

Lowercase letters denote different alleles amplified by the primer pair.

The P2 marker revealed five different alleles, among which *P. ginseng* and *P. notoginseng* had two specific alleles, whereas *P. quinquefolium* had one allele 'c' (Fig. 1A). In the capillary electrophoresis diagram of P2 primer, *P. ginseng, P. quinquefolium*, and *P. notoginseng* could be distinguished by peak shape, height, and fragment size. The highest peak of *P. ginseng* first appeared. However, the highest peak of *P. quinquefolium* appeared at the latest. The number of fragments of *P. notoginseng* was the largest. The results of capillary electrophoresis were consistent with those of native-PAGE (Fig. 1H). Therefore, two methods for testing EST-SSR polymorphism could be mutually confirmed. The other six pairs of primers all had specific alleles for species identification, and the PCR products all conformed to their predicted sizes, ranging from 100 to 400 bp (Fig. 1).

3.2 Identification of commercial products using EST-SSR markers

Seven pairs of EST-SSR markers were used to identify fifty commercial products, including thirty *P. ginseng* products (ten ginseng shredded slices, ten ginseng powders, and ten red ginseng slices), ten *P. quinquefolium* slices, and ten *P. notoginseng* powders. Using fresh samples as standard, the SSR standard fingerprint was constructed. The SSR polymorphic bands of commercial products were compared with the SSR standard fingerprint to determine the raw materials of the products. In the detection process of commercial ginseng products, the amplification efficiency of red ginseng slices was

the lowest. This result was related to the breakage of DNA fragments caused by high-temperature treatment, so it is necessary to increase the number of amplification cycles (Fig. 2C, E, and F). Among the seven pairs of EST-SSR primers, the combination of P2 and Q11 primers showed the best identification effect. The detection rates of *P. ginseng, P. quinquefolium*, and *P. notoginseng* were 90%, 85%, and 98% respectively. Generally, our identification system has successfully identified commercial *P. ginseng, P. quinquefolium*, and *P. notoginseng* products.

3.3 Identification of garden ginseng, forest ginseng and wild ginseng using EST-SSR markers

Seven pairs of EST-SSR primers were used in five *Panax* species, namely garden ginseng, forest ginseng, wild ginseng, *P. quinquefolium*, and *P. notoginseng*. The discrepancy of EST-SSR polymorphism among garden ginseng, forest ginseng, and wild ginseng was much less than that of *P. ginseng, P. quinquefolium*, and *P. notoginseng*. Moreover, polymorphism was gradually decreased in the three ginseng cultivars, followed by garden ginseng, forest ginseng, and wild ginseng, which was related to the age and environment of ginseng. Taking P2 and P8 markers as examples, the P2 revealed five different alleles. Alleles ‘b’ and ‘d’ were specific for *P. ginseng*. ‘d’ was the co-appearance of garden ginseng, forest ginseng, and wild ginseng allele. But ‘b’ was getting shallower in three ginseng cultivars (Fig. 3A). The Q8 marker revealed similar conclusions. ‘b’ and ‘c’ alleles were unique to *P. ginseng.* ‘b’ was the co-dominant allele of garden ginseng, forest ginseng, and wild ginseng. However, ‘c’ was getting shallower among the three ginseng cultivars (Fig. 3B). Other primers showed the same regularity in three ginseng varieties (Fig. 3C-F).

In the experiment, P35 and Q2 markers could be used to specifically identify garden ginseng, forest ginseng, and wild ginseng. In the native-PAGE of P35 marker, garden ginseng had one allele, while forest ginseng and wild ginseng had two alleles. The EST-SSR polymorphism gradually decreased in three ginseng cultivars, which was consistent with the general regularity of EST-SSR polymorphism (Fig. 3G). In the capillary electrophoresis pattern of P35 primer, there was only one peak in garden ginseng, while two peaks in forest ginseng and wild ginseng. The peak occurrence time was consistent with the result of native-PAGE (Fig. 3I). In the Q2 primer, there were three specific alleles in wild ginseng at 800 bp, but two alleles in garden ginseng and forest ginseng (Fig. 3H).

3.4 Evaluation on phylogenetic relationship of panax species

Phylogenetic analysis was conducted with UPGMA clustering using genotypes of seven pairs of EST-SSR markers for identifying genetic relationships among 10 individuals from the five *Panax* Species, each species two plants (Fig. 4). The genetic relationship between *P. ginseng* and *P. quinquefolium* was closer than that of *P. notoginseng*, while the genetic relationship between forest ginseng and garden ginseng was closer than that of wild ginseng.

4. Discussion

At present, DNA molecular markers are widely used in the identification of medicinal plants, such as *Velvet Antler, Astragalus membranaceus, Ganoderma lucidum, Codonopsis pilosula*, and so on (Yuan et al. 2016; Na et al. 2004; Kwon et al. 2019; Guo et al. 2007). Many DNA molecular markers have been applied to identify *P. ginseng, P. quinquefolium*, and *P. notoginseng*. These DNA markers can divide into three parts: molecular hybridization-based polymorphism, PCR-based polymorphism, and direct DNA sequence analysis. Ngan et al. (1999) identified six *Panax* species from two common adulterants by RFLP. Based on molecular hybridization technology, the limitation of RFLP is that it needs a large number of samples to extract enough DNA, and the operation process is time-consuming and laborious. Polymorphism analysis methods based on PCR include RAPD, AFLP, and SSR. Um et al. (2001) and Shaw et al. (1995) used RAPD markers to distinguish ginseng from its adulterants, but this method has limited reproducibility and low resolution. Choi et al. (1008) used AFLP markers to identify *P. ginseng*, *P. quinquefolium*, and *P. notoginseng*. While this technique is sensitive and specific, it does require two steps: amplification followed by restriction enzyme digestion. Direct DNA sequence analysis mainly includes SNP markers. Liu et al. (2016) successfully applied SNP on *ITS2* to identify *P. quinquefolium* in decoctions, Chinese patent medicines, and other ginseng products. SNP markers can identify differences between single nucleotides, which may have the potential to show genetic variation, but it is hard to solve the problem of non-specific amplification. These markers are not sufficient to meet the needs of cultivars authentication and molecular breeding.

In contrast, SSR markers have the characteristics of abundant quantity, good repeatability, uniform distribution, and high polymorphism (Taheri et al. 2018; Vu et al. 2020). In the research of *Panax* species, most of the articles focused on the screening of SSR primers, and few use SSR markers to identify *P. ginseng, P. quinquefolium*, and *P. notoginseng.* We selected seven pairs of SSR primers to identify three subspecies of ginseng and found that the polymorphism difference between *P. ginseng* and *P. quinquefolium* was smaller than that of *P. notoginseng*, which was similar to the genetic relationship obtained by RAPD markers cluster analysis (Shaw et al. 1995). In the identification of commercial ginseng products, we found that the combination of primers P2 and Q11 was the best. In the process of testing, the detection rate of *P. notoginseng* was the highest, which may be due to the simple process of the products, resulting in less degradation of DNA fragments. Compared with ginseng shredded slices and powders, the detection rate of red ginseng was lower, which may be due to the serious gene breakage caused by high-temperature processing. The number of PCR cycles should be appropriately increased, which is similar to identifying ginseng products by SNP markers (Jung et al. 2014).

Currently, the identification of garden ginseng, forest ginseng, and wild ginseng is mainly based on morphology. However, this method needs long-term experience and is greatly affected by subjective factors, making it difficult to establish reliable identification criteria (Wu et al. 2018). Besides morphological identification, the diversity in chemical properties is mainly due to the difference in content and composition of ginsenoside. The content of rare ginsenoside in wild ginseng is significantly higher than cultivated ginseng, while the content of common ginsenoside in cultivated ginseng is higher. In addition, the volatile components of wild ginseng also have obvious differences (Liu et al. 2016), indicating that the chemical components of wild ginseng are different from cultivated ginseng, which is

related to the age and growth environment of ginseng. The disadvantages of chemical analysis are that the instrument is expensive and the sample pretreatment process is tedious, making it difficult to perform comparative analysis in large quantities. The three ginseng varieties also have significant differences in gene expression. Studies showed that the genes of *pGAPDH-w*, *p-psbB*, and *NRT2* in wild ginseng were up-regulated, which indicated the particularity of wild ginseng (Han et al. 2013; Kim et al. 2012; Kwon et al. 2011).

Because the genome and transcriptome information of *Panax* species is incomplete. There are few studies on garden ginseng, forest ginseng, and wild ginseng by using DNA molecular markers (Wang et al. 2016). Some research showed SSR markers could reveal intraspecies level variation, such as variation among ginseng cultivars and individuals (Vu et al. 2020; Choi et al. 2011; Kim et al. 2012). However, most articles of SSR markers only pay attention to the identification of ginseng cultivars in Korea (Choi et al. 2011; Kim et al. 2012), and there are no reports on the differentiation of three Chinese ginseng varieties, garden ginseng, forest ginseng, and wild ginseng. In this study, the EST databases in NCBI were compared by using the method of bioinformatics, and seven pairs of SSR markers were screened out. SSR polymorphism gradually reduced in three Chinese ginseng Cultivars, and a specific band of primer Q2 was screened out in wild ginseng. This trend might be due to garden ginseng and forest ginseng coming from a few wild ginseng populations. Besides, this certain difference may be inherited. Bai et al. (1997) used RAPD markers to analyze wild and cultivated *P. quinquefolius*. The results showed that the range of genetic distance in cultivated populations was lower than that of wild populations, which was proposed to be due to selection by ginseng farmers. Choi et al. (2011) designed and developed the 70 EST-SSR markers conformed to Mendelian inheritance rules in Yunpoong and Chunpoong, indicating these SSR markers were replicable and could be stably inherited. Kim et al. (2012) successfully utilized an EST-SSR marker set to identify two ginseng cultivars among 70 ginseng individuals and to select true F1 hybrid plants.

Compared with the third generation molecular marker SNP, SSR has the advantages of less non-specificity and better repeatability, which is beneficial to the identification of ginseng products. However, the development of traditional SSR markers is time-consuming, laborious, and cumbersome. With the establishment and application of EST database, the development of SSR markers has become simple, and efficient, and is closely related to gene coding regions. At present, simple, rapid, and automatic capillary electrophoresis technology can be applied to the detection of SSR markers, making the identification of SSR markers more accurate and efficient. Our research results showed that the results of native-PAGE and capillary electrophoresis were consistent and could confirm each other.

Seven pairs of EST-SSR primers were selected to identify *P. ginseng, P. quinquefolium, P. notoginseng*, and their commodities. In the testing of commercial products, the combination of primers P2 and Q11 had the highest positive rate. In three ginseng cultivars (garden ginseng, forest ginseng, and wild ginseng), we found that SSR polymorphism gradually decreased, which may be related to the age and growth environment of ginseng. Two pairs of SSR primers can specifically distinguish three ginseng subspecies. The SSR molecular markers selected in this experiment have high repeatability and accuracy, which is

expected to provide new ideas for the identification of three ginseng varieties and lay a foundation for standardizing the ginseng market.

Declarations

Acknowledgements Thanks to National Ginseng Product Quality Supervision and Inspection Center and Jilin Yisheng Hansen Biotechnology Co., Ltd. for providing experimental sample.

Author's contributions Data collection: Xin-yuan FAN, Li-mei LIU; design of the study: Xin-yuan FAN, He-tong HUI, Tian-qi WANG; statistical analysis: Feng-he SU, Lun-peng WU; analysis and interpretation of the data: Xin-yuan FAN, Cheng-ri LIN; drafting the manuscript: Xin-yuan FAN, He-tong HUI; critical revision of the manuscript: Li-mei LIU

Funding This research was supported by Jilin province the Science and Technology Development Plan Project (20190304108YY).

Compliance with ethical standards

Conflflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

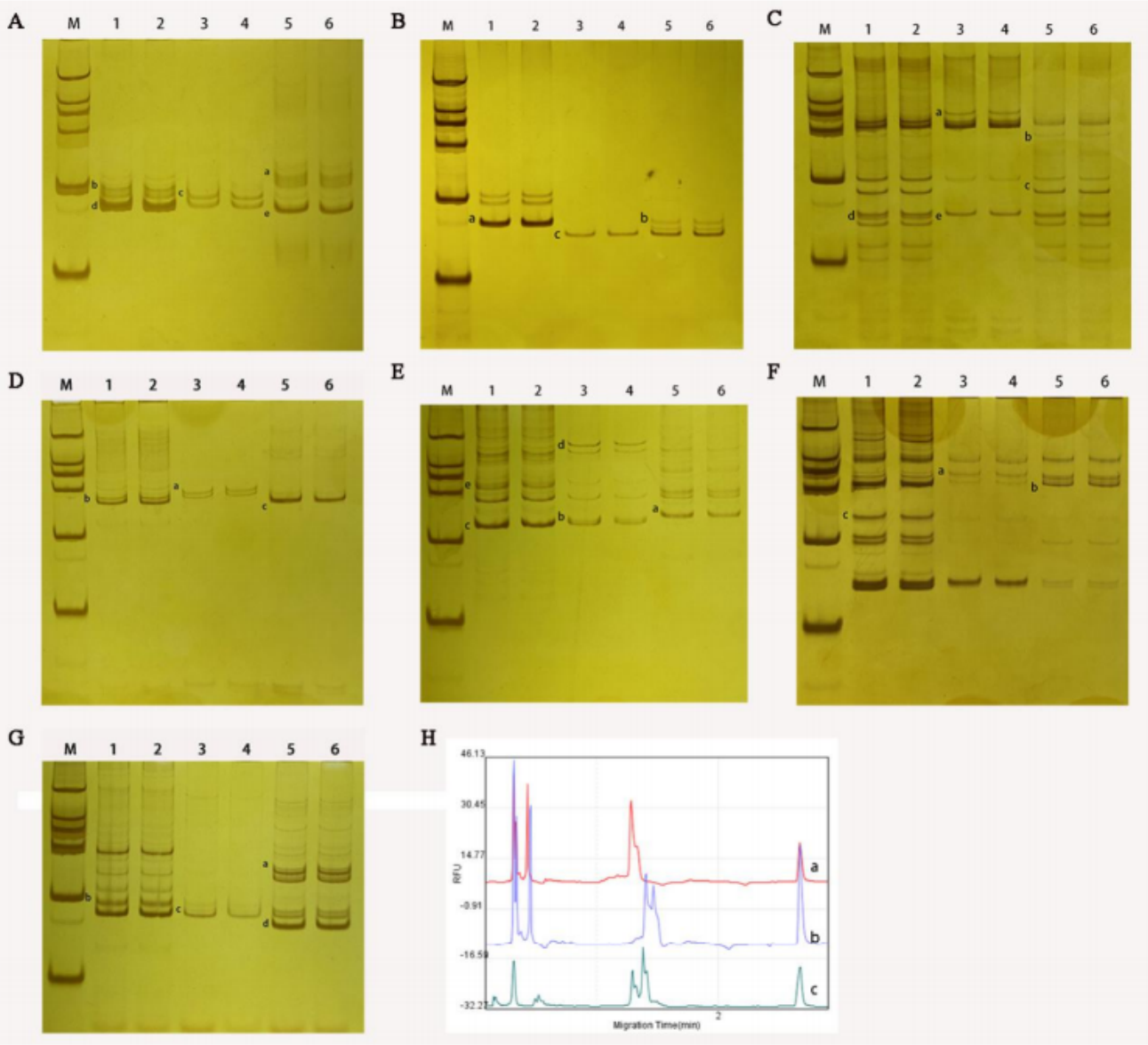

Figure 1

Figure 1

Authentication of Panax ginseng, Panax quinquefolium and Panax notoginseng by 7 EST-SSR Markers. (A-G) are band profiles which were amplified by primer pairs P2, P35, Q2, Q3, Q5, Q8 and Q11, respectively. M:D2000bp DNA ladder; Lanes:1-2, Panax ginseng; 3-4, Panax quinquefolium; 5-6, Panax notoginseng. Lowercase letters denote different alleles amplified by the primer pair. (H) Primer P2 capillary electrophoresis. a-b are Panax ginseng, Panax quinquefolium and Panax notoginseng, respectively.

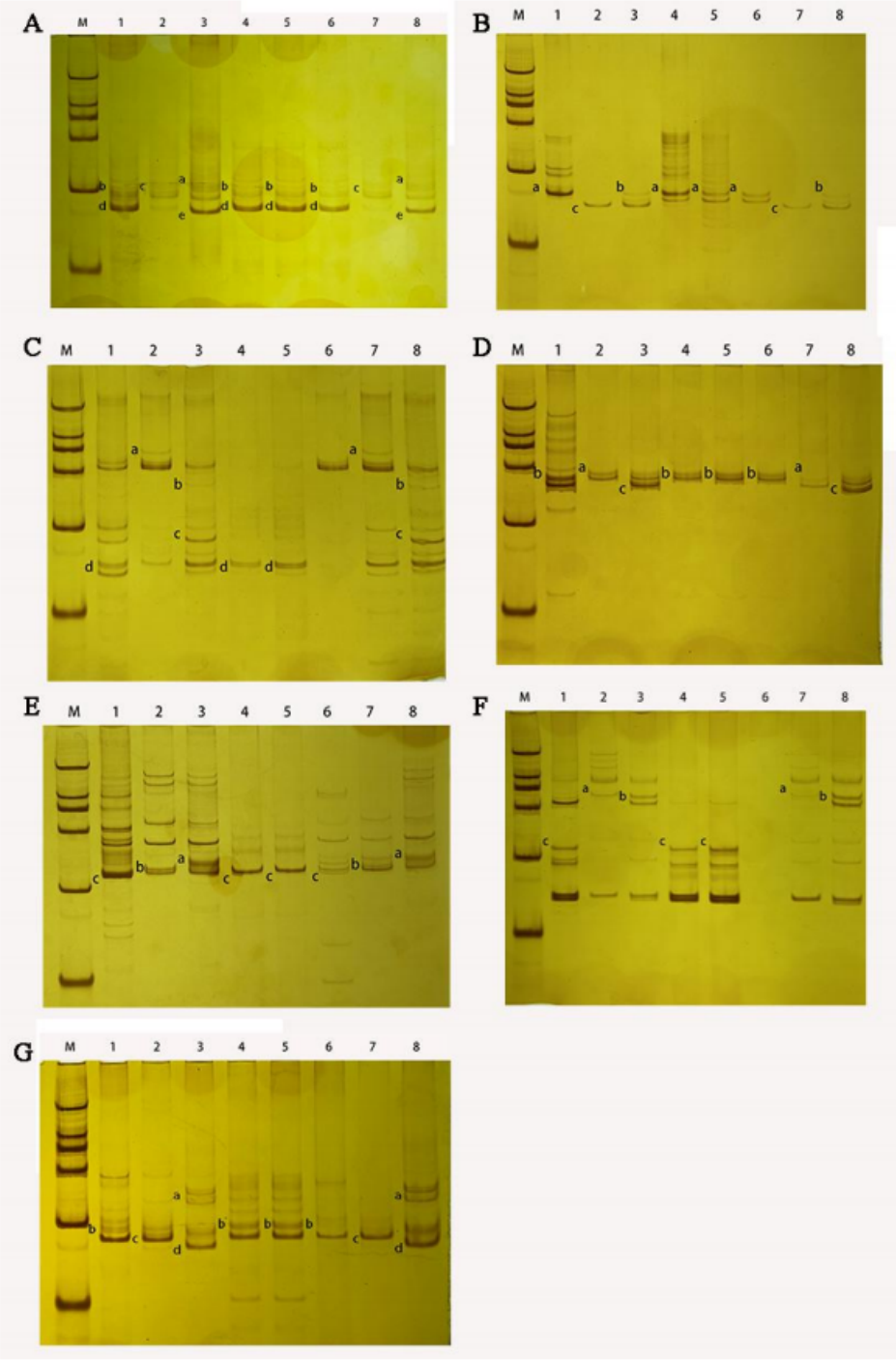

Figure 2

Figure 2

Authentication of commercial ginseng products based on EST-SSR markers. (A-G) are band profiles which were amplified by primer pairs P2,P35,Q2,Q3,Q5,Q8 and Q11, respectively. M, D2000bp DNA ladder; Lanes:1-3, Standard fingerprint of Panax ginseng, Panax quinquefolium and Panax notoginseng；4, ginseng shredded slices；5, ginseng powders；6, red ginsengs；7, quinquefolium shredded slices；8, notoginseng powders. Lowercase letters denote different alleles amplified by the primer pair.

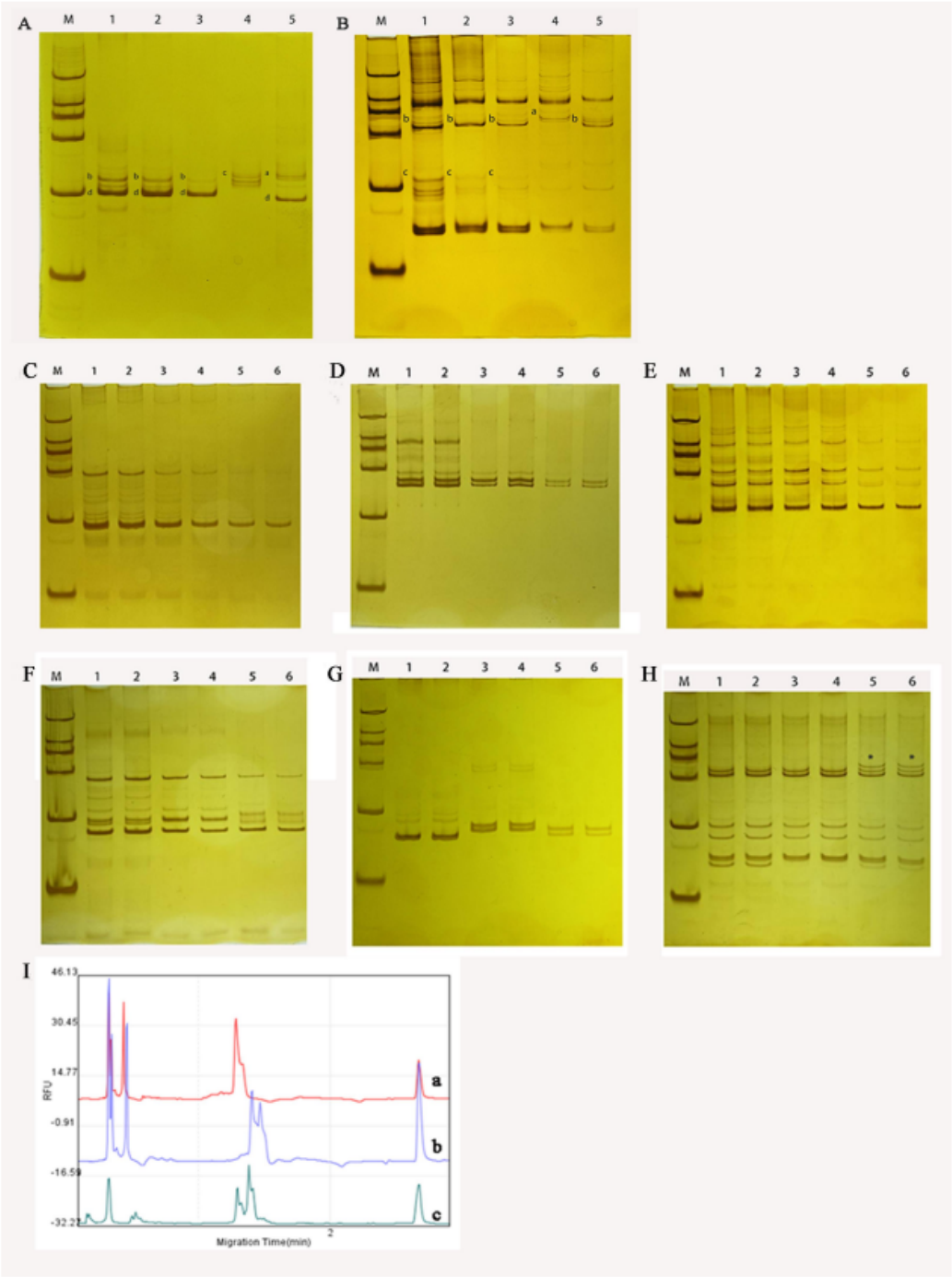

Figure 3

Figure 3

Polymorphism of EST-SSR markers in garden ginseng, forest ginseng, wild ginseng, Panax quinquefolium and Panax notoginseng. B) are band profiles which were amplified by primer pairs P2 and Q8, respectively. M:D2000bp DNA ladder; Lanes:1, garden ginseng; 2, forest ginseng; 3, wild ginseng; 4, Panax quinquefolium; 5, Panax notoginseng. (C-H) are band profiles which were amplified by primer pairs P17, Q5, Q11, Q3, P35 and Q2 respectively. M:D2000bp DNA ladder; Lanes:1-2, garden ginseng; 3-4, forest

ginseng; 5-6, wild ginseng. *was the specific band of wild ginseng. (I) Primer P35 capillary electrophoresis. a-b are garden ginseng, forest ginseng and wild ginseng, respectively.

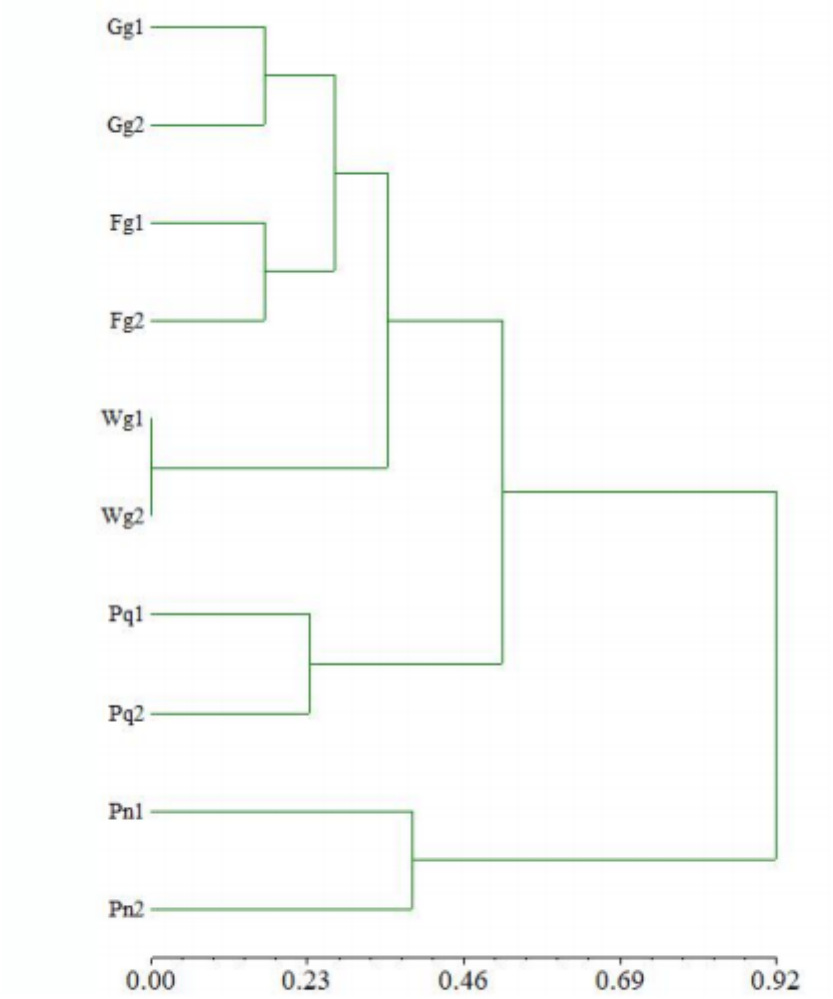

Figure 4

Figure 4

Phylogenetic analysis of Panax species. Analysis includes two individuals from each cultivar: Gg, garden ginseng; Fg, forest ginseng; Wg, wild ginseng; Pq, Panax quinquefolium; Pn, Panax notoginseng.