



Contents lists available at ScienceDirect

## Journal of Ginseng Research

journal homepage: <http://www.ginsengres.org>

## Review article

Applications of molecular markers in the discrimination of *Panax* species and Korean ginseng cultivars (*Panax ginseng*)Ick Hyun Jo<sup>1</sup>, Young Chang Kim<sup>1</sup>, Dong Hwi Kim<sup>1</sup>, Kee Hong Kim<sup>1</sup>, Tae Kyung Hyun<sup>2</sup>, Hojin Ryu<sup>3,\*</sup>, Kyong Hwan Bang<sup>4,\*</sup><sup>1</sup> Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, Rural Development Administration, Eumseong 27715, South Korea<sup>2</sup> Department of Industrial Plant Science and Technology, Chungbuk National University, Cheongju 28644, South Korea<sup>3</sup> Department of Biology, Chungbuk National University, Cheongju 28644, South Korea<sup>4</sup> Department of Planning and Coordination, National Institute of Horticultural and Herbal Science, Rural Development Administration, Wanju 55365, South Korea

## ARTICLE INFO

## Article history:

Received 14 May 2016

Accepted 19 September 2016

Available online xxx

## Keywords:

high-resolution melting

*Panax ginseng*

molecular marker

single-nucleotide polymorphism

## ABSTRACT

The development of molecular markers is one of the most useful methods for molecular breeding and marker-based molecular associated selections. Even though there is less information on the reference genome, molecular markers are indispensable tools for determination of genetic variation and identification of species with high levels of accuracy and reproducibility. The demand for molecular approaches for marker-based breeding and genetic discriminations in *Panax* species has greatly increased in recent times and has been successfully applied for various purposes. However, owing to the existence of diverse molecular techniques and differences in their principles and applications, there should be careful consideration while selecting appropriate marker types. In this review, we outline the recent status of different molecular marker applications in ginseng research and industrial fields. In addition, we discuss the basic principles, requirements, and advantages and disadvantages of the most widely used molecular markers, including restriction fragment length polymorphism, random amplified polymorphic DNA, sequence tag sites, simple sequence repeats, and single nucleotide polymorphisms.

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## 1. Introduction

Korean ginseng (*Panax ginseng*) is one of the most favored herbal medicinal plants valued by local Korean consumers as well as those based overseas. The growth condition of Korean ginseng cultivation requires careful monitoring system of the ideal cultivar conditions in its natural habitat; owing to this as well as the stringent cultivation and processing techniques such as those required in the manufacture of red ginseng (hongsam). Furthermore, it is considered superior to the cultivars of competing ginseng producing countries [1–3]. However, recently, foreign ginseng, which is cheaper than Korean ginseng and is of questionable safety, has been illegally distributed as a Korean domestic produce, thereby hindering the development of the ginseng industry [4,5]. In addition,

relatively inexpensive local varieties with less uniform seeds are being misleadingly packaged as new varieties, putting cultivators of genuine new varieties at a disadvantage [6]. Consequently, it is becoming difficult for domestic cultivators to obtain guaranteed stable profits. As consumer trust in domestic produce declines, domestic ginseng producers face considerable losses.

An effort to ensure distinctness and seed uniformity during the seed production stage is necessary to counter this problem. International institutions such as the World Trade Organization, Trade Related Intellectual Property Rights, Organization for Economic Cooperation and Development, and the European Union have made the protection of intellectual property rights for domestically produced varieties compulsory. Moreover, with the establishment of the International Union for the Protection of New Varieties of Plants

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<http://dx.doi.org/10.1016/j.jgr.2016.09.001>

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(UPOV), various institutional devices are being developed to protect rights related to plant varieties [7]. In its endeavor to protect domestic and foreign intellectual property rights for domestic varieties, Korea has joined the UPOV, to ensure the international profits and rights of breeders of newly developed varieties. Furthermore, the Korea Seed and Variety Service has put forward clear criteria for establishing varieties. In order to protect the new domestic varieties, this institution assesses for the cultivars distinctness, uniformity, and stability [8].

A comparative assessment of external morphology was performed to test the distinctness of new varieties. However, the differences that determine distinctness are often qualitative, which makes it difficult to accurately distinguish varieties based on morphology alone [9]. Accordingly, biochemical and molecular biological techniques have recently been applied. Molecular markers are especially advantageous because they are not affected by the cultivation environment, such as the soil conditions, and because large samples can be analyzed quickly and accurately in the laboratory [10–12]. Molecular markers are now being used frequently to distinguish cultivars of ginseng. Therefore, in this review article, we summarize the current knowledge regarding the development of molecular markers, and provide a focused review of their recent application for genetic diversity analysis of *Panax* species and Korean ginseng cultivars.

## 2. Molecular markers for discrimination in *Panax* species

### 2.1. Polymerase chain reaction-restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) is a method that is based on differences in the length of DNA fragments obtained when DNA from different sources is digested with restriction enzymes that recognize specific sequences (Fig. 1). Although RFLP has been suggested as an important tool for genome mapping and genetic fingerprinting, it requires a large amount of sample DNA

and probe, which is a labeled DNA sequence that hybridizes with one or more fragments. Therefore, in order to overcome the disadvantages associated with RFLP, polymerase chain reaction (PCR)-RFLP based on endonuclease digestion of PCR-amplified DNA has been introduced as an extremely valuable tool for genotyping of species-specific variations.

Because of the simplicity and speed of procedures and the requirement of small amounts of DNA, this technique has been widely utilized for species identification and taxonomic research. Based on the differences in the 18S rRNA gene sequence, three species in the *Panax* genus can be differentiated at the DNA level by PCR-RFLP and mutant allele specific amplification [13]. Ngan et al [14] conducted an RFLP analysis of ribosomal ITS1-5.8S-ITS2 sequence among six species belonging to the *Panax* genus and a ginseng mixture; specifically, these researchers differentiated *P. ginseng* and *Panax quinquefolius*. This indicates that the sequence homology within the ITS1 and ITS2 regions is low in these two species. In addition, Um et al [15] carried out random amplified polymorphic DNA (RAPD) and PCR-RFLP and obtained DNA fingerprints that are different among individual ginseng plants. Yang and Kim [16] cleaved ginseng chlorophyll *psbA* and *rbcL* genes by restriction enzymes for differentiation and analyzed interspecies and intraspecies mutational variation. Furthermore, distinguishing Korean ginseng from various mixtures of medicinal plants could be possible by gradient PCR and RFLP applications [17].

Genetic analyses of Korean and foreign ginseng cultivars by Kim et al [18] were performed and successfully distinguished the cultivars Gopoong and Gumpoong (Korean ginseng cultivars). In addition, Diao et al [19] used PCR-RFLP and an amplification refractory mutation system based on the 5S rDNA sequence from commercially available complex drug mixtures and proved the existence of *P. ginseng*. All of the studies reviewed were able to differentiate the plants only at the species level and analyzed only a limited number of plant samples; collectively, these studies were unable to distinguish all cultivars. Thus, PCR-RFLP shows good reproducibility in the discrimination of species in the ginseng genus but the discriminatory ability of PCR-RFLP for cultivars differentiation is low.

### 2.2. RAPD

The RAPD method is relatively simpler than RFLP and can easily identify polymorphic DNA fragments (Fig. 2). Because the method requires only small amounts of DNA, RAPD can also be used for

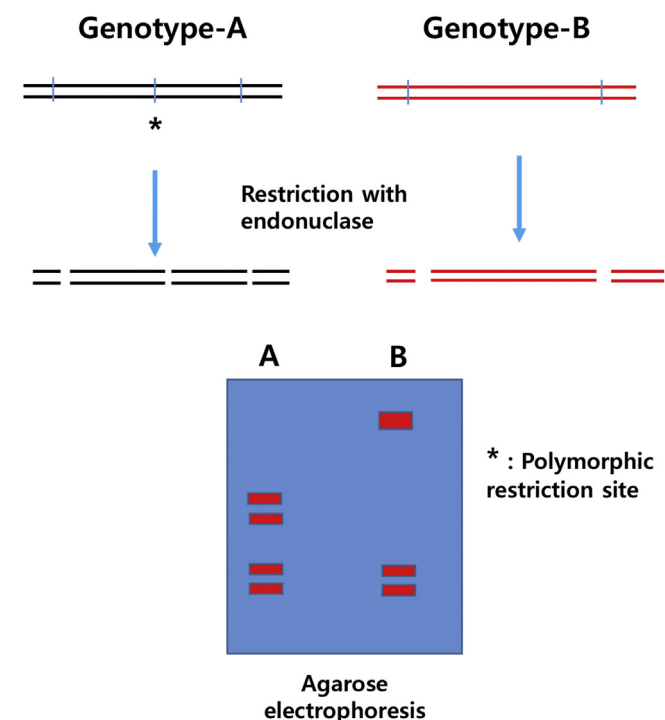


Fig. 1. Schematic explanation of restriction fragment length polymorphism.

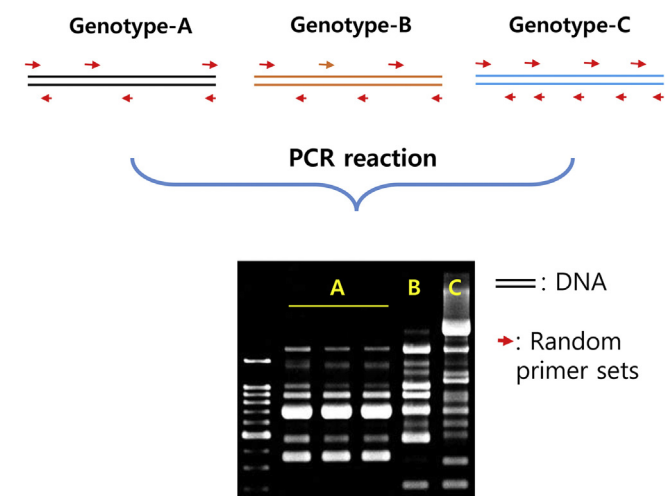


Fig. 2. Schematic explanation of random amplified polymorphic DNA.

testing of genetic characteristics of many individuals, thus making it a highly economical method [20,21]. However, RAPD is a dominant marker and cannot be used for segregation of the F2 population. This method uses a short primer, approximately a 10mer, resulting in relatively low reproducibility. Initially, Um et al [15] reported the possibility of classification of ginseng species by comparing interspecies and intraspecies mutational relations by means of polymorphic bands of RAPD. Based on the genetic diversity in American ginseng cultivated in Ontario, two more species could be identified by using RAPD analysis [22], and their results suggested that it is possible to improve the genetic improvement in ginseng cultivars. Shao et al [23] produced 18 random primers for RAPD analysis of ginseng, studied the genetic diversity of the *P. quinquefolius* population in North America, and identified population specific RAPD markers [24]. Moreover, 25 primers with high reproducibility, from 130 random primers, were developed by Bang et al. [25], and used these primers to study the genetic diversity among *Panax* species and cultivars of Korean ginseng [25].

At present, RAPD analysis of ginseng is broadly used for elucidating species-level genetic differences or for genetic diversity analysis of cultivars due to lower efficiency in discrimination of cultivars [26–28]. Moreover, RAPD generates nonspecific amplicons depending on experimental and ambient conditions; therefore, it is difficult to ensure reproducibility. To overcome this, an analysis of a sequence-characterized amplified region (SCAR) marker as the most effective method in terms of convenience and reproducibility among the various molecular biological methods was examined for identification of ginseng species and discrimination of cultivars [29], suggesting that a SCAR marker can be used to distinguish three species: *P. ginseng*, *P. quinquefolius*, and *Panax notoginseng*, and the Korean ginseng cultivar Sunone.

### 2.3. Sequence-tagged site

The sequence-tagged site (STS) method was developed by Olson et al [30] and involves analysis of a sequence for specific clones followed by construction of diverse primers for genetic analysis. A DNA library needs to be established for the production of specific STS primer sets [31]. Recently, the methylation filtering (MF) method, which removes methylated repetitive DNA from a library, has become a popular method that can increase cloning frequency of genomic regions [32]. The MF method enables removal of repetitive DNA and is thus useful for creating a library of genetic regions [33,34]. Bang et al [31] built a genomic DNA library from the Yunpoong cultivar of the Korean ginseng by using the MF method; furthermore, on the basis of this information, they identified and reported eight STS markers that can differentiate six cultivars of Korean ginseng by using 208 pairs of STS primers. Several novel STS markers were developed by Jo et al [9], and were able to apply to this approach to 11 cultivars of Korean ginseng to identify a marker capable of distinguishing the new cultivar Cheonryang.

RAPD and PCR-RFLP markers show insufficient numbers of suitable loci within the genome and have poor sensitivity, reliability, discriminatory ability, and reproducibility, posing difficulties in terms of discrimination of strains [35]. In contrast, an STS marker with the simplicity of its analytical method allows for easy confirmation of results with high reproducibility and, therefore, can be applied to the analysis of massive genetic resources and to cultivar discrimination [36,37].

### 2.4. Simple sequence repeats

Simple sequence repeats (SSR) analysis involves the fabrication of primers for repeated monomeric, dimeric, trimeric, and

tetrameric DNA sequences (Fig. 3). This method is relatively expensive and time consuming for creation of a genomic library and for primer fabrication; however, it involves a codominant marker and has very high reproducibility [38,39]. SSR markers are reported to be effective for strain discrimination because they cover multiple alleles and have genomic region specificity, high polymorphism, and reproducibility [40,41].

The ginseng SSR marker, although developed relatively late in comparison with other markers, has been actively used in recent years. Kim et al [42] and Ma et al [43] produced a ginseng microsatellite-enriched genomic library and identified a microsatellite sequence through screening of the library. Useful microsatellite sequences during end-sequence analysis of a Bacterial artificial chromosome (BAC) genomic library were also isolated and applied [44]. In addition, the developed SSR markers were successfully applied to American ginseng (*P. quinquefolius*) discrimination. However, Jo et al [45] reported no significant difference in the allele distribution of microsatellite sequences between ginseng cultivars and wild ginseng. Research performed by Hon et al [46] revealed that Korean ginseng (*P. ginseng*) and American ginseng (*P. quinquefolius*) could be distinguishable from each by analyzing the microsatellite sequences identified in the latter.

Recently, an alternative strategy has been developed that uses chloroplast genome, Expressed sequence tag (EST) and transcriptome information to identify SSR markers. Kim and Lee [47] reported 18 SSR markers that show species-specific mutations in the ginseng chlorophyll genes. Moreover, 68 sets of SSR markers from 7,055 expressed sequence tags of *P. ginseng* developed as powerful markers that effectively distinguish between the Chinese cultivars of *P. ginseng*, 'Ji'anhangbo' and 'Fusong'ermaya' [48]. Interestingly, Xu et al [49] used sequence-related amplified polymorphism analysis to study the genetic diversity of *P. ginseng* cultivated in China. Also, 19 EST-SSR markers to distinguish nine cultivars of Korean ginseng was proposed as an effective method for cultivar discrimination [50]. As described above, the SSR markers have been widely used in studies of genetic diversity of ginseng, analysis of genetic relations, and cultivar discrimination because of high polymorphism and reproducibility. However, this method has its limitations in that the analysis is time-consuming and because of the use of polyacrylamide gels requires skilled operators.

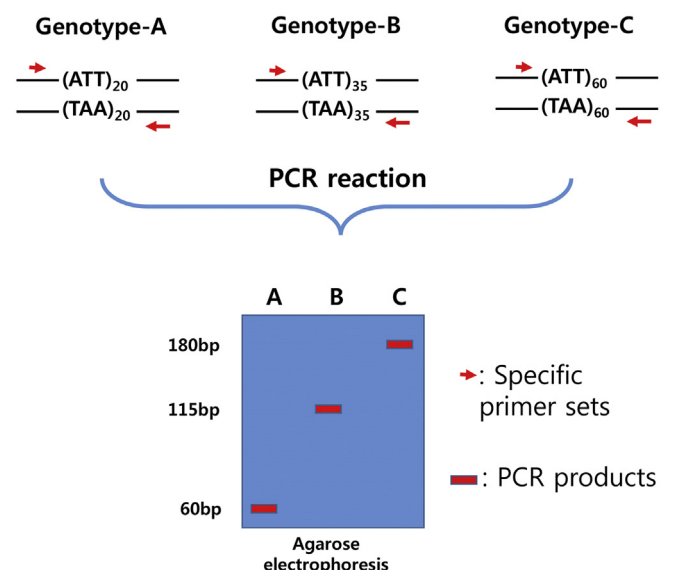
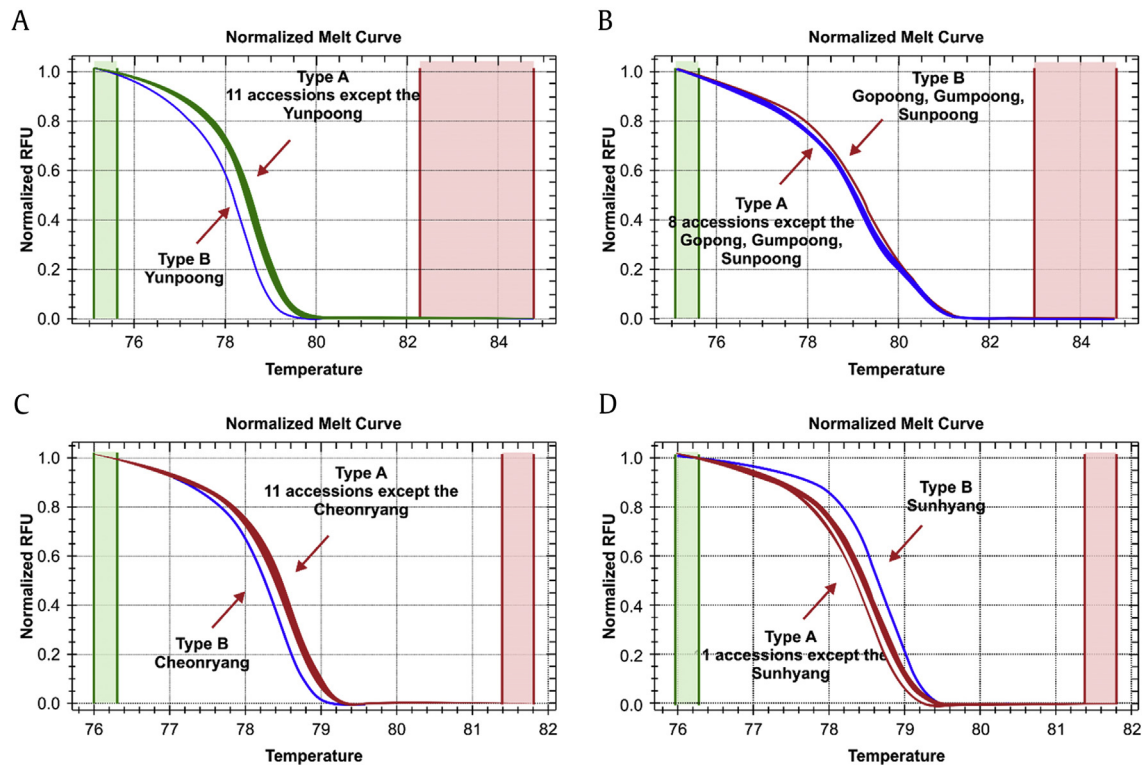


Fig. 3. Schematic explanation of simple sequence repeat marker.



**Fig. 4.** Examples of rapid discrimination of *Panax ginseng* cultivars and *Panax quinquefolius* by use of high-resolution melt analysis with (A) GHP 01095, (B) GHP 11494, (C) GHP 13830, and (D) GHP 15673 markers. Temperature unit is °C [Jo et al [54], 2015].

### 2.5. Single nucleotide polymorphisms

A single nucleotide polymorphism (SNP) is a single-base mutation in DNA and most frequently affects the genome of all somatic cells and has the advantage of being a stable analytical parameter [51]. Wang et al [52] examined the SNPs of mitochondrial cytochrome oxidase subunit 2, regions of introns 1 and 2, and could successfully develop a specific marker for Chunpoong, a popular cultivar in Korea. Yunpoong-specific SNP in the exon of the glyceraldehyde 3-phosphate dehydrogenase gene was identified and this polymorphism was able to distinguish this cultivar from the major ginseng cultivars in Korea [53]. Jo et al [6] produced three pairs of TaqMan-MGB probes for an SNP that is present in genomic DNA to perform genotyping; thus, they developed a method for discrimination of Korean ginseng (Fig. 4). Recent developments in next-generation sequencing technology (NGS) have accelerated the growth of sequencing information. It is now possible to elucidate a large number of SNPs using transcript information. Consistent with this trend, Jo et al [54] identified a large number of SNPs in the transcript information on ginseng and developed high-resolution melting (HRM) analysis that can distinguish cultivars (Fig. 4). Various research groups have successfully attempted species and cultivar discrimination by SNP genotyping by using HRM analysis. For example, the use of SNP genotyping with HRM analysis was usefully applied for discrimination among almond cultivars or *citrus* species [55,56].

In the HRM method, an intercalating fluorescent dye that is added during PCR binds to amplicons, and is analyzed for changes in characteristics of the fluorescent signals dependent on a temperature increase and the sequence of the target regions [57,58]. Therefore, HRM analysis allows for more rapid and convenient discrimination of PCR target regions based on SNP variation, thus making it possible to distinguish cultivars by genotyping of DNA samples.

### 3. Conclusion and future perspectives

In order to prepare for opening of the market by free-trade agreements affecting the ginseng industry, competing countries, including China, the United States, and Canada, intend to deal with the problem of illegal outflow of Korean ginseng species. It is necessary, therefore, to select DNA markers that can distinguish cultivar and to establish a database using the selected markers. In Korea, currently, research and development of ginseng cultivar is conducted in a highly competitive industry, which has also to compete with produce from other countries such as China, the United States, and Canada. For this, scientific certification technology for the development of excellent cultivars and systematic species management needs to be implemented. The lack of this certification would be a big setback to the progress of the Korean ginseng industry. The problems the ginseng industry will be faced with can be listed as follows: (1) a case where domestic cultivars are illegally shipped overseas and are imported back to Korea and sold as domestic product; (2) a case where native species are used to create new cultivars for the relevant domestic markets and these cultivars are sold at higher prices; and (3) a case when poor management of species during cultivation leads to a species of lowered genetic purity.

Conventional methods of DNA analysis are inconvenient, expensive, and time-consuming with respect to strain discrimination. These methods are also limited by the low frequency of polymorphisms in *Panax* species. In recent years, identification of standard genomic information and acceleration of massive production of sequence data by means of NGS has allowed for easy and rapid identification of mutations such as SNP, SSR, and Insertion/Deletion (IN/DEL) in full-length genomes. As such, the NGS method offers big benefits at low costs and is rapidly facilitating identification of genetic markers in ginseng plants. Diverse DNA markers



like RFLP, RAPD, STS, SSR, and SNP analyses are being developed for discrimination of ginseng cultivar.

In conclusion, as described in this review, the development of useful efficient markers must be established. This information would be beneficial for basic ginseng breeding methods. The markers once identified and established could offer analysis of consistent results and highlight the importance of preserving genetic distinctness, uniformity, discrimination of promising line during ginseng breeding, and identification of the parent plants

### Conflicts of interest

The authors have no conflicts of interest with any parties or individuals.

### Acknowledgments

This work was carried out with the support of the Basic Science Research Program through the National Research Foundation of Korea (2015R1A4A1041869) Korean Ministry of Science, ICT and Future Planning and Cooperative Research Program for Agriculture Science and Technology Development (PJ01047301), Rural Development Administration, Korea.

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