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ORIGINAL ARTICLE

# Differentiation of Black Raspberry Fruits According to Species and Geographic Origins by Genomic Analysis and $^{1}$H-NMR-based Metabolic Profiling

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**Abstract** Random amplification of polymorphic DNA (RAPD) and NMR techniques were used to differentiate and identify species of black raspberry (BR) of different geographic origins. BR leaf samples from five geographic origins were identified as *Rubus japonicus* and *R. coreanus* by RAPD. $^{1}$H-NMR analysis was also performed for BR fruit extracts from different geographic origins. Major compounds assigned in $^{1}$H-NMR spectra of BR fruits were amino acids, organic acids, sugars, phenolic acids, and purine derivatives. In addition, relative levels of total phenolic compounds, flavanols, flavonoids, and anthocyanins in the BR fruits were further analyzed. Hierarchical cluster analyses (HCA) based on the genetic (RAPD of leaf samples) and metabolic ($^{1}$H-NMR, total phenolic compounds, flavanols, flavonoids and anthocyanins of fruit samples) datasets were independently performed. The HCA dendrogram pattern derived from RAPD genetic fingerprinting of BR leaf samples matched that from the $^{1}$H-NMR data of BR fruit samples. This research demonstrates that metabolic profiling of BR fruit based on $^{1}$H-NMR is a promising method for differentiating BR fruits of various species and geographic origins.

**Keywords** black raspberry · geographic origin · $^{1}$H-NMR · metabolomics · random amplification of polymorphic DNA

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## Introduction

The black raspberry (BR) belongs to the genus *Rubus* and the family *Rosaceae*, and the fruits are used as folk medicine and food resource. International interest in growing and producing BR is increasing due to its well established antioxidant, antiinflammatory, antibiotic, antiviral, and immune modulation activities (Kim and Lee, 1991; Kim et al., 1997; de Ancos et al., 2000; Kim et al., 2000; Lee and Do, 2000; Kwon et al., 2007). The therapeutic effects of the BR fruits may be related to its bioactive phytochemical constituents, such as flavonoids, anthocyanins, tannins, triterpenoids, phenolic acids, and organic acids (Yoon et al., 2003; Tian et al., 2006). It is hypothesized that the contents of total phenolic compounds, such as flavanols, flavonoids and anthocyanins in BR fruits vary according to species and geographical origins.

Random amplification of polymorphic DNA (RAPD) is a polymerase chain reaction (PCR)-based technique that amplifies random DNA fragments using single short primers of arbitrary nucleotide sequence (Lee et al., 2007). The technique has been extensively applied to several areas of plant research, such as identifying cultivars of the calla lily (*Zantedeschia* spp.) (Hamada

and Hagimori, 1996), assessing genetic diversity in caster (*Ricinus communis* L.) (Gajera et al., 2010), determining mutations due to gamma radiation in soybean plants (*Glycine max* L. Merrill) (Atak et al., 2004), and confirming genetic integrity in radish (*Raphanus sativus* L.) varieties (Kwak et al., 2009). DNA analysis enables genomic fingerprinting with consequent identification of different individuals. In the agro-food industry, such procedures are thought to have interesting applications for identifying species and cultivars of both raw materials and processed food (Pasqualone et al., 2004).

NMR techniques coupled with multivariate data analysis have recently been applied to metabolic profiling and determining the geographical origin of plant materials and food sources (Anastasiadi et al., 2009; Jung et al., 2010; Lee et al., 2010; Longobardi et al., 2012). The term 'metabolomics' describes a comprehensive analysis of the metabolic state of a biological system under a given set of conditions at a particular time point (Fiehn et al., 2000). Previous studies have used metabolomics techniques based on $^1$H-NMR to model relationships among active components and $^1$H-NMR spectra in BR fruits (Wyzgoski et al., 2010), and to monitor metabolic changes in BR fruits during maturation (Kim et al., 2011). There have been no reports regarding the genetic and metabolic differentiation of different species and geographical origins of BR samples.

We hypothesized that BR fruits from different species or geographical origins have characteristic metabolic information, and there might be a close relationship between genomic and metabolic information. In general, genetic fingerprinting experiment such as RAPD is performed using leaves rather than fruit samples, because clearer results could be obtained from leaf samples than those from fruits. In the present study, genetic analysis of BR leaf samples was performed using RAPD and analyzed the metabolic information ($^1$H-NMR data, contents of total phenolic compounds, flavanols, flavonoids and anthocyanins) of BR fruits to investigate correlation between genomic and metabolic information of BR leaves and fruits of various geographical origins. The objective of the study was to differentiate BR fruits of different species and geographical origins by metabolic information.

## Materials and Method

**Plant materials.** Ripe BR fruits and leaves from each cultivar were harvested from a local farm in the Geochang, Gochang, Sunchang, Jeongeup, Jinan, Gokseong, and Hoengseong provinces, Korea, in 2009. The origin of each cultivar is shown in Fig. 1. Fruit and leaf samples were harvested between July 10$^{th}$ and 20$^{th}$. Supplemental Table 1 lists the daily mean temperature ($^o$C), maximum temperature ($^o$C), minimum temperature ($^o$C), and daily average relative humidity (%), sun-exposure time (h), and monthly total rainfall (h) during the harvest period for each of the seven provinces that provided BR samples.

The most common *Rubus* species native to the Republic of Korea is *Rubus coreanus* Miquel. Although *Rubus japonicus* is native to Japan, it is also grown in the Republic of Korea. Authentic leaf samples from *R. japonicus* and *R. coreanus* used for RAPD experiments were obtained and identified by The National Arboretum of the Republic of Korea. The fruit surfaces were immediately cleaned with distilled water, and the samples were freeze-dried and then stored at −70$^o$C before analysis. Voucher specimens were deposited at the College of Pharmacy, Chung-Ang University, Korea (CAURU 20090801–20090832).


**Fig. 1** Cultivation and harvesting locations of BR samples in the Republic of Korea.

**Chemicals and reagents.** Chloroform, methanol, potassium dihydrogen phosphate, and ethylacetate were obtained from Daejung Chemicals & Metals Co., Ltd. (Korea). Primers were purchased from JK Biotech. Co. (Korea). HQ *Taq* polymerase was acquired from Roche Molecular Diagnostics (USA) and a 1-kb marker was obtained from Life Technologies (USA). NaOD was purchased from Cortec (France). All other reagents including BLOTTO (Bovine Lacto Transfer Technique Optimizer), deuterium oxide [($D_2O$, D 99.9 atom %), containing 0.05% of 3-(trimethylsilyl)-propionic-2,2,3,3-$d_4$ acid sodium salt (TSP)], $NaN_3$, and Folin-Ciocalteu reagent were from Sigma (USA).

**RAPD using BR leaves.** In plants with high polyphenol content, such as BR, any contaminating polyphenol compounds in the PCR can degrade DNA (John, 1992) and react with the $Mg^{2+}$ in the PCR solution, thereby inhibiting the polymerase. De Boer et al. (1995) reported that BLOTTO prevents polyphenols from inhibiting PCR. Yoo et al. (2001) also used BLOTTO to achieve successful performance of PCR on BR.

DNA was extracted from fresh leaf samples cut to 0.5 cm$^2$. Fresh leaf samples (0.1 g) obtained by collecting and pooling 10–20 leaves from each cultivar were ground in liquid nitrogen, transferred to an Eppendorf tube, and 400 μL of lysis buffer (100 mM NaCl, 0.5 M Tris-HCl (pH 8.0), 10% SDS) was added to each sample and mixed thoroughly. One microliter of proteinase

**Table 1** Assignment of $^{1}$H-NMR spectral peaks of BR fruits obtained from (a) Gokseong and (b) Gochang (s: singlet, d: doublet, t: triplet, m: multiplet, dd: doublet of doublet)

| # | Compound name | Origin | Chemical shift (peak pattern, *J* value) |
|---|---|---|---|
| | **Amino acids** | | |
| 1 | Isoleucine | (a) | 0.90 (t, *J*=6.82), 0.96 (d, *J*=7.23) |
| | | (b) | 0.90 (t, *J*=7.71), 0.97 (d, *J*=6.76) |
| 2 | Valine | (a) | 0.96 (d, *J*=7.23), 1.02 (d, *J*=6.92), 2.24–2.29 (m) |
| | | (b) | 0.97 (d, *J*=6.76), 1.02 (d, *J*=7.30), 2.24–2.29 (m) |
| 3 | Alanine | (a) | 1.46 (d, *J*=7.21) |
| | | (b) | 1.46 (d, *J*=7.23) |
| 4 | Citrulline | (a) | 1.48–1.55 (m), 1.88–1.98 (m) |
| | | (b) | 1.50–1.55 (m), 1.95–2.01 (m) |
| 5 | Arginine | (a) | 1.59–1.66 (m), 1.73–1.81 (m), 1.89–1.95 (m) |
| | | (b) | 1.60–1.66 (m), 1.70–1.78 (m), 1.89–1.96 (m) |
| 6 | Leucine | (a) | 1.67–1.73 (m) |
| | | (b) | 1.69–1.76 (m) |
| 7 | Proline | (a) | 1.95–1.99 (m), 2.30–2.37 (m) |
| | | (b) | 1.97–2.07 (m), 2.30–2.36 (m) |
| 8 | Glutamic acid | (a) | 2.08–2.15 (m), 2.42–2.50 (m) |
| | | (b) | 2.11–2.15 (m), 2.40–2.48 (m) |
| 9 | Glutamine | (a) | 2.08–2.16 (m), 2.45–2.50 (m) |
| | | (b) | 2.10–2.17 (m), 2.40–2.48 (m) |
| 10 | Asparagine | (a) | 2.83–2.89 (m), 2.94–2.99 (m) |
| | | (b) | 2.74–2.80 (m), 2.88–2.93 (m) |
| 11 | Glycine | (a) | 3.55 (s) |
| | | (b) | 3.55 (s) |
| 12 | Tyrosine | (a) | 6.85–6.88 (m), 7.20–7.22 (m) |
| | | (b) | 6.86–6.89 (m), 7.17–7.20 (m) |
| 13 | Tryptophan | (a) | 7.16–7.19 (m), 7.28 (s), 7.55 (d, *J*=7.65), 7.69 (d, *J*=6.97) |
| | | (b) | 7.16–7.20 (m), 7.28 (s), 7.54 (d, *J*=7.66), 7.70 (d, *J*=6.53) |
| | **Organic acids** | | |
| 14 | 3-hydroxybutyric acid | (a) | 1.21 (d, *J*=6.81), 2.44–2.48 (m) |
| | | (b) | 1.21 (d, *J*=7.10), 2.42–2.48 (m) |
| 15 | Lactic acid | (a) | 1.31 (d, *J*=6.63) |
| | | (b) | 1.31 (d, *J*=6.67) |
| 16 | Acetic acid | (a) | 2.06 (s) |
| | | (b) | 2.06 (s) |
| 17 | Pyruvic acid | (a) | 2.35 (s) |
| | | (b) | 2.38 (s) |
| 18 | Succinic acid | (a) | 2.63 (s) |
| | | (b) | 2.62 (s) |
| 19 | Citric acid | (a) | 2.85 (d, *J*=17.92), 2.96 (d, *J*=17.88) |
| | | (b) | 2.77 (d, *J*=14.84), 2.90 (d, *J*=13.68) |
| 20 | Ascorbic acid | (a) | 3.72–3.76 (m), 3.97–4.01 (m) |
| | | (b) | 3.72–3.77 (m), 4.00–4.03 (m) |
| 21 | Tartaric acid | (a) | 4.36 (s) |
| | | (b) | 4.35 (s) |
| 22 | Maleic acid | (a) | 6.25 (s) |
| | | (b) | 6.30 (s) |
| 23 | Formic acid | (a) | 8.35 (s) |
| | | (b) | 8.44 (s) |

K (50 mg/mL) was added and incubated at 37°C for 1 h. Subsequently, 400 µL of 2 × cetyltrimethylammonium bromide (CTAB) containing 2% CTAB (w/v), 10 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, and 1% polyvinyl pyrrolidone

**Table 1** Continued

| # | Compound name | Origin | Chemical shift (peak pattern, *J* value) |
|---|---|---|---|
| | **Sugars** | | |
| 24 | Glucose | (a) | 3.22 (dd, $J_1$=9.22, $J_2$=8.12), 3.35–3.41 (m), 3.42–3.48 (m), 3.51 (dd, $J_1$=9.93, $J_2$=3.67), 3.68–3.75(m), 3.78–3.82 (m), 3.87 (dd, $J_1$=11.96, $J_2$=1.59), 4.62 (d, *J*=7.96), 5.21 (d, *J*=3.73) |
| | | (b) | 3.22 (dd, $J_1$=9.71, $J_2$=7.91), 3.35 – 3.41 (m), 3.42–3.48 (m), 3.51 (dd, $J_1$=9.97, $J_2$=3.70), 3.72–3.76 (m), 3.79–3.82 (m), 3.87 (dd, $J_1$=10.72, $J_2$=2.79), 4.62 (d, *J*=7.96), 5.21 (d, *J*=3.68) |
| 25 | Sucrose | (a) | 4.19 (d, *J*=8.75), 5.39 (d, *J*=3.79) |
| | | (b) | 4.20 (d, *J*=8.29), 5.39 (d, *J*=3.83) |
| 26 | Fructose | (a) | 3.51–3.59 (m), 3.65–3.70 (m), 3.76–3.82 (m), 3.87 (dd, $J_1$=10.26, $J_2$=3.41), 3.96–4.02 (m), 4.08 (d, *J*=3.57) |
| | | (b) | 3.51–3.58 (m), 3.63–3.71 (m), 3.76–3.82 (m), 3.87 (dd, $J_1$=10.72, $J_2$=2.79), 3.96–4.02 (m), 4.09 (d, *J*=3.63) |
| | **Phenolic compounds** | | |
| 27 | Epicatechin | (a) | 5.00–5.03 (m), 6.15 (d, *J*=2.07), 6.16 (d, *J*=2.16) |
| | | (b) | 5.00–5.02 (m), 6.10 (d, *J*=2.45), 6.12 (d, *J*=2.91) |
| 28 | Protocatechuic acid | (a) | 6.92 (d, *J*=8.63) |
| | | (b) | 6.92 (d, *J*=7.50) |
| 29 | Vanillic acid | (a) | 7.43–7.46 (m) |
| | | (b) | 7.42–7.44 (m) |
| | **Purine derivatives** | | |
| 30 | 3-methylxanthine | (a) | 7.56 (s) |
| | | (b) | 7.55 (s) |
| 31 | Xanthine | (a) | 7.79 (s) |
| | | (b) | 7.69 (s) |
| 32 | Hypoxanthine | (a) | 8.10 (s), 8.11 (s) |
| | | (b) | 8.09 (s), 8.10 (s) |

(PVP) was added to the mixture and incubated at 65°C for 1 h. After incubation, 600 µL of phenol:chloroform:isoamylalcohol (25:24:1) was added and centrifuged at 14,000 rpm for 10 min. The resulting supernatant was carefully transferred to a new Eppendorf tube. To the supernatant, a 0.7 volume of isopropanol was added, and the mixture was stored at room temperature for 10 min to precipitate the DNA. The pelleted DNA precipitate was washed with 500 µL of 70% ethanol and centrifuged at 14,000 rpm for 5 min. The pellet was vacuum-dried and then dissolved in 50–100 µL of TE-buffer containing 1 M Tris-HCl (pH 8.0) and 0.5 M EDTA. The solution was incubated with 3 µL of RNAse A (10 mg/mL) at 37°C for 1 h to remove residual RNA, and the sample was stored at –20°C until use.

Preliminary RAPD-PCR was conducted to optimize the RAPD-PCR conditions. Among the tested conditions, RAPD with 1% BLOTTO (10% skim milk power [Fluka, Switzerland] and 0.2% $NaN_3$) in the PCR solution, UPFs 2 and 3 and 13 primers (20 pmole, JK Biotech. Co., Korea), and 3 mM of $MgSO_4$ yielded the best results, and were used for all subsequent analyses. After screening the initial primers using arbitrary decamer oligonucleotide primers (UPF series), three potentially useful primers revealed high levels of DNA polymorphism among the isolates and were used for RAPD of BR leaf samples.

Amplification reactions consisted of 100 ng of DNA, 5 µL of 10 × HQ Taq buffer ($Mg^+$ free) (100 mM Tris-HCl [pH 8.0], 500 mM KCl, 0.01% gelatin), 3 mM $MgSO_4$, 1 µL each of 2.5 mM dATP, dCTP, dGTP and dTTP, 1 µL of UPF primers, 0.5 µL of HQ *Taq* polymerase (Boehringer, Germany), and 1% BLOTTO. All PCR reactions were incubated at 94°C for 4 min followed by 35 cycles of 1 min at 94°C for denaturation, 1 min at 44°C for annealing, and 3 min at 72°C for extension using a PCR machine (PTC-200, MJ Research, USA). The final extension period was 7 min at 72°C.

The amplification products were loaded on a 1.5% agarose gel using TAE buffer (45 mM Tris-acetate, 1 mM EDTA, pH 8.0), and a 1-kb marker was included. Ten microliters of each PCR product was mixed with loading buffer and loaded on the gel. Gels were visualized by staining with ethidium bromide solution (0.5 µg $mL^{-1}$). The gel was imaged under UV light using a red filter.

The positions of reproducible and consistent RAPD bands were scored and transformed into a binary character matrix ("1" for the presence and "0" for the absence of RAPD band at a particular position). A phylogenetic tree was created by the unweighted pair group method arithmetic (UPGMA) average cluster analysis using the output data and the graphical module of the NTSYS-pc software (version 2.2, Exeter software, USA).

**$^1$H-NMR spectrometry of BR fruits.** Thirty-two individual BR

fruit samples (five samples from each cultivar except the two samples from Hoengseong) were extracted by adding 5 mL of 50% methanol to 100 mg of freeze-dried and powdered BR in a centrifuge tube, vortexed for 1 min, and sonicated for 1 min. The materials were then centrifuged at 500 × g for 20 min. The extracts were transferred to a 50-mL round-bottomed flask. The extraction procedure was performed two times on each sample followed by concentration under vacuum rotary evaporation (Eyela, Japan). $KH_2PO_4$ (1.232 g) was added to 100 mL of $D_2O$ [containing 0.05% TSP as an internal standard for $D_2O$] as a buffering agent. The pH of the $D_2O$ used for NMR measurements was adjusted to 6.0 using 520 μL of 1 N NaOD. Extracts were dissolved in 1 mL of $D_2O$ and then transferred to a 5-mm NMR tube.

$^1$H-NMR spectra were recorded at a temperature of 298 K on a 600.13-MHz Bruker Avance spectrometer (Bruker Biospin, Germany) using a broadband observe (BBO) probe. A zgcppr pulse sequence was applied to suppress the residual water signal. A total of 128 transients were collected into 64K data points with a relaxation delay of 1 s. A spectral width of 11363.6 Hz and an acquisition time per scan of 3.04 s were used. Prior to Fourier transformation, an exponential line broadening function of 0.3 Hz was applied to the free induction decay.

MestReNova (version 6.0.4, Mestrelab Research, Spain) was used to obtain the NMR spectra, which were all automatically encoded in ASCII files using AMIX (version 3.7, Bruker BioSpin, USA) software. The spectral $^1$H-NMR region from δ=0.54 to δ=10.00 was segmented into regions with widths of 0.04 ppm, giving 230 integrated regions in each NMR spectrum. The 4.70–5.00 ppm region was excluded to remove water resonance. $^1$H-NMR signal assignments were performed by comparing their chemical shifts and splitting patterns to the Chenomx NMR suite software (version 5.1, Chenomx, Canada) and HMDB (open access database, http://hmdb.ca). Each spectral intensity dataset was normalized to the total sum of the spectral regions. The resulting datasets were then converted to Microsoft Office Excel (version 2007, Microsoft, USA) format and imported into SIMCA-P+ software (version 12.0, Umetrics, Sweden) for partial least squares-discriminant analysis (PLS-DA) and hierarchical cluster analysis (HCA). PLS-DA can yield a clearer differentiation of each class and enable a less complicated investigation of marker compounds than principal component analysis (PCA) by rendering the class to each sample group (Eriksson et al., 2006).

In HCA, single linkage was used as a similarity or distance criterion (Hartigan, 1985), and Euclidean distance was chosen as a distance measure between a pair of clusters after performing preliminary test using various similarity or distance criteria (data not shown). Significant differences in metabolite levels were detected by independent t-test followed by correction via Levene's tests (Carroll and Schneider, 1985) for equality of variances using SPSS software (version 18, SPSS Inc., USA). The level of statistical significance was set at $p$ <0.05.

**Analysis of polyphenolic, anthocyanin, flavonoid, and flavonol contents of BR fruits.** Polyphenolic extraction was performed according to the method described by Quettier-Deleu et al. (2000). Ten grams of powdered BR fruit sample was macerated for 24 h at 4°C in 4×400 mL of methanol:acetone:$H_2O$ (7:7:3, v/v/v), and the crude extracts were filtered. The filtrates were concentrated under low-pressure at 30°C, and the remaining aqueous phase was exhausted by ethyl acetate. The ethylacetate phase was evaporated, and the residue was dissolved in methanol:$H_2O$ (2:8, v/v). Additionally the methanol was eliminated by evaporation, and the water phase was freeze-dried. For further assays, the freeze-dried powder was dissolved in appropriate solvents. The ethanol fraction was used to estimate total phenolic and anthocyanin contents, and the methanol fraction was used for all other chemical analysis. Generally, 2 mg powdered sample/mL solvent was used.

Total phenolic content was determined using a modified Folin-Ciocalteu method of Singleton and Rossi (1965). Each test sample (200 μL) was added to a 10-mL test tube containing 2.6 mL distilled water. After vortexing the tubes, 200 μL Folin-Ciocalteu's phenol reagent was added to each tube. The tubes were vortexed, and 6 min later, 2 mL of 7% $Na_2CO_3$ was added to each tube. The tubes were vortexed again and allowed to stand for 90 min at room temperature. The absorbance of each sample was measured against a blank at 750 nm using a spectrophotometer (Optizeu 2120UV, Mecasys, Korea). Total phenolic content is expressed as mg of gallic acid equiv/100 g dry sample.

Flavanol content was measured by comparison with a (-) epicatechin standard treated with the same procedure mention above. One milliliter of the extraction dissolved in methanol was added to 5 mL of 0.1% (w/v) ρ-dimethylaminocinnamaldehyde in methanol:HCl (3:1, v/v) reagent (Porter et al., 1985). The absorbance was measured 10 min later at 640 nm, and the results were expressed in mg of (-) epicatechin equivalents/100 g dry sample.

Total flavonoid content was determined using a modified version of the method described by Zhishen et al. (1999). To each test sample in a 10-mL volumetric flask, 4 mL of distilled water was added. Five min after adding 0.3 mL of 5% $NaNO_2$, 0.3 mL of 10% $AlCl_3$ was added. After 6 min, 2 mL of 1 M NaOH were added, and the volume was brought up to 10 mL with distilled water. The absorbance of the solution was measured against a blank at 510 nm using a spectrophotometer (Optizeu 2120UV). The total flavonoid content was expressed as milligrams of catechin per gram of dry extract. All measurements in the present study were performed in triplicate, and the data are reported as mean values ± standard deviation.

Anthocyanins were extracted according to the method described by Abdel-Aal and Hucl (1999). Ten milligrams of powdered samples were extracted with 24 mL of acidic ethanol (ethanol:1.0 N HCl, 85:15, v/v) and adjusted to pH 1 with 4N HCl. The final solution was shaken for 15 min at 150 rpm, then readjusted to pH 1 and shaken for an additional 15 min, if necessary. The tube was

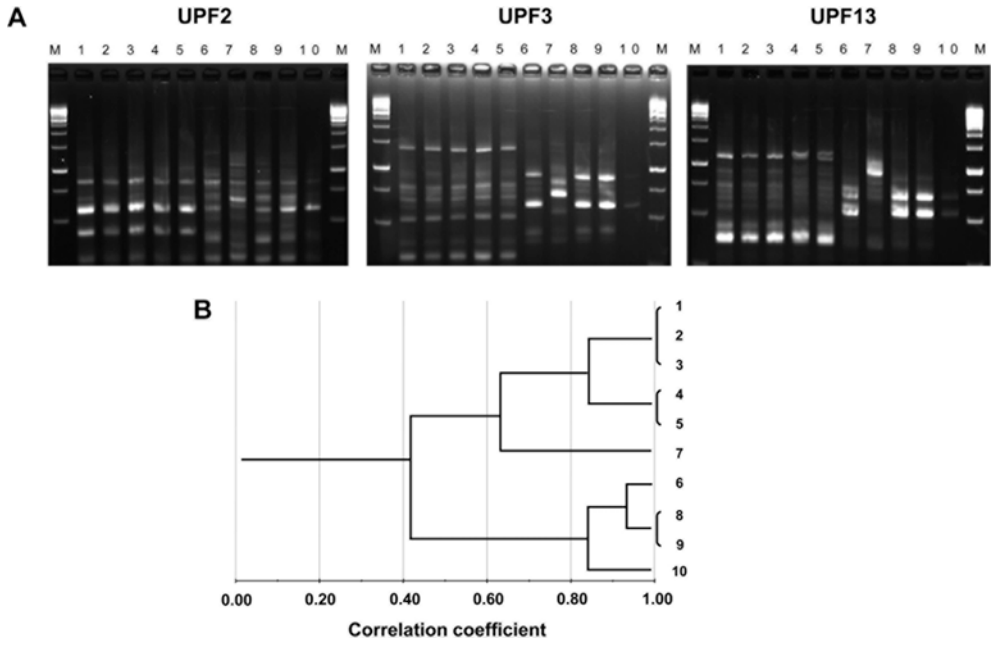


**Fig. 2** Random amplified polymorphic DNA patterns of BR leaves of different origins using UPF2, UPF3, and UPF13 primers (A), and dendrogram derived from RAPD (B). M: λ *Hin*dIII marker, 1: Geochang BR, 2: Gochang BR, 3: Sunchang BR, 4: Jeongeup BR, 5: Jinan BR, 6: Gokseong BR, 7: *Rubus japonicus* identified by The National Arboretum of the Republic of Korea, 8: Hoengseong, 9: *Rubus* species identified by The National Arboretum of the Republic of Korea, 10: *Rubus coreanus* identified by The National Arboretum of the Republic of Korea.

centrifuged at 27,200 × g for 15 min, the supernatant was poured into a 50-mL volumetric flask, and brought up to final volume with acidic ethanol. The absorbance was measured at 535 nm against a reagent blank. The results are expressed in grams cyanidin 3-glucoside/100 g dry sample by comparison with standard cyanidin 3-glucoside treated with the same procedure.

With the polyphenolic, anthocyanin, flavonoid, and flavonol data, PLS-DA and HCA were performed using SIMCA-P+ software (version 12.0, Umetrics). In HCA, single linkage was used as a similarity or distance criterion (Hartigan, 1985), and Euclidean distance was used as the distance measure between a pair of clusters.

## Results and Discussion

**RAPD analysis of BR leaves.** Fig. 2a shows the bands corresponding to DNA amplified using the UPF primers. The phylogenetic tree (Fig. 2b) was created by the UPGMA average gene cluster analysis, and the dendrogram revealed two clusters. Cluster 1 had two accessions, samples 1, 2, 3, 4, 5, and 7 from Geochang, Gochang, Sunchang, Jeongeup, Jinan, and authentic *R. japonicus,* respectively. In addition, cluster 2 contained two accessions of samples 6, 8, 9, and 10 from Gokseong, Hoengseong and authentic *R. coreanus,* respectively. These results suggest that samples from Geochang, Gochang, Sunchang, Jeongeup, and Jinan are likely *R. japonicus,* whereas the samples from Gokseong and Hoengseong as *R. coreanus*. The unidentified *Rubus* species, 9, was also assumed to be *R. coreanus* based on the dendrogram.

**Assignment of the peaks in $^{1}$H-NMR spectra and comparison of relative levels of assigned compound.** Supplemental fig. 1 shows a representative NMR spectrum of the BR samples from Gokseong (a) and Gochang (b). BR fruit from each origin had similar peak intensities in the 06 ppm region. In the 69 ppm region, however, fruits from Gokseong had lower overall peak intensities than those from Gochang.

Detailed peak assignments for the metabolites were performed based on comparison with the Chenomx NMR software suite database (Table 1). These assignments included amino acids (isoleucine, valine, alanine, citrulline, arginine, leucine, proline, glutamic acid, glutamine, asparagines, glycine, tyrosine, and tryptophan); organic acids (3-hydroxybutyric, lactic, acetic, pyruvic, succinic, citric, ascorbic, tartaric, maleic, and formic); sugars (glucose, sucrose, and fructose); phenolic compounds (epicatechin, protocatechuic, and vanillic acids); and purine derivatives (3-methylxanthine, xanthine, and hypoxanthine). The compounds assigned in the 69 ppm region were phenolic compounds and purine derivatives. An independent t-test was performed to compare the concentration of each compound from each sample. According to the HCA dendrogram derived from RAPD, samples of each origin were divided into two groups: *R. coreanus* (samples from Gokseong and Hoengseong) and *R. japonicus* (samples from Geochang, Gochang, Jeongeup, Sunchang and Jinan). The sugar levels (i.e., glucose and fructose) and organic acids (i.e., ascorbic acid and succinic acid) were

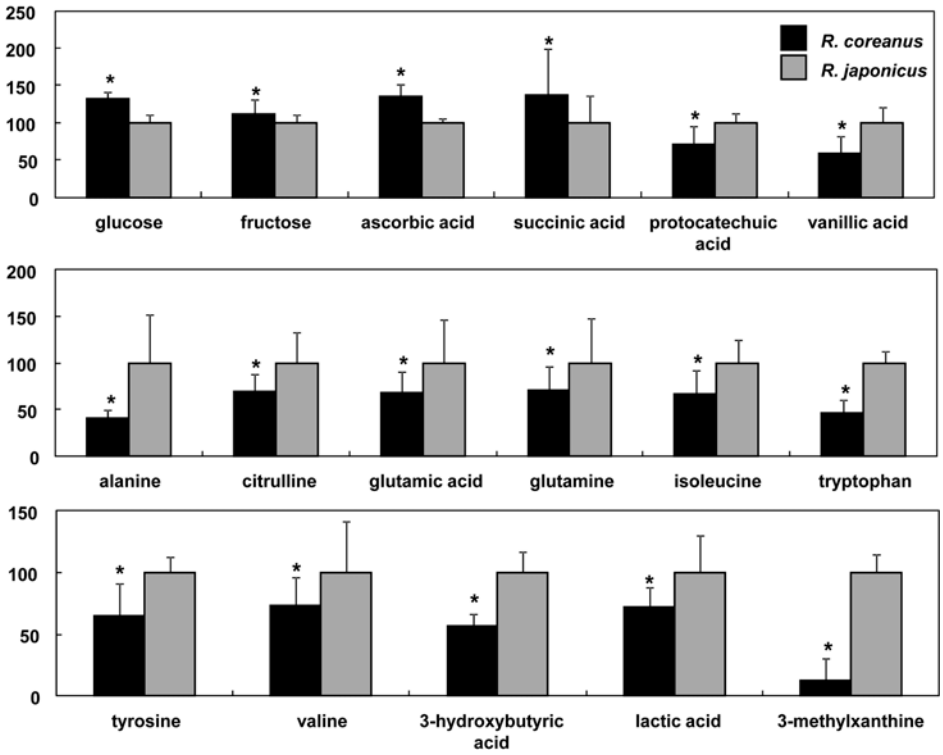


**Fig. 3** The relative levels of major compounds in BR fruits. Group 1: Geochang, Gochang, Sunchang, Jinan, Jeongeup; Group 2: Gokseong, Hoengseong. *indicates the statistical difference at $p$ <0.05).

**Table 2** Total phenolic, flavanol, flavonoid, anthocyanin, and flavonoid contents of BR fruits of different geographical origins. Each value represents the mean ± SD. Each superscript (a, b, c, and d) in the same column represents statistical difference at $p$ <0.05.

| Cultivation location | Total phenol (g/100g dried weight) | Flavanol (g/100g dried weight) | Flavonoid (g/100g dried weight) | Anthocyanin (g/100g dried weight) |
|---|---|---|---|---|
| Geochang | 0.54±0.146$^{bc}$ | 0.09±0.011$^{cd}$ | 0.13±0.003$^{b}$ | 2.90±0.15$^{a}$ |
| Gochang | 0.67±0.091$^{c}$ | 0.09±0.013$^{c}$ | 0.16±0.006$^{c}$ | 3.01±0.24$^{d}$ |
| Sunchang | 0.69±0.127$^{c}$ | 0.10±0.016$^{b}$ | 0.13±0.010$^{b}$ | 2.93±0.28$^{c}$ |
| Jeongeup | 0.38±0.133$^{a}$ | 0.04±0.008$^{b}$ | 0.19±0.019$^{d}$ | 2.81±0.21$^{a}$ |
| Jinan | 0.45±0.088$^{ab}$ | 0.05±0.005$^{b}$ | 0.12±0.021$^{b}$ | 2.27±0.24$^{b}$ |
| Gokseong | 1.05±0.141$^{d}$ | 0.03±0.002$^{a}$ | 0.17±0.014$^{c}$ | 1.55±0.13$^{a}$ |
| Hoengseong | 0.67±0.161$^{c}$ | 0.03±0.004$^{a}$ | 0.10±0.009$^{a}$ | 1.88±0.17$^{a}$ |

significantly higher in *R. coreanus* than in *R. japonicas* (Fig. 3). In addition, levels of amino acids (i.e., isoleucine, valine, alanine, citrulline, glutamic acid, glutamine, tyrosine and tryptophan), organic acids (i.e., lactic acid and 3-hydroxybutyric acid), phenolic compounds (i.e., protocatechuic acid and vanillic acid), and purine derivatives (i.e., 3-methylxanthine) were significantly lower in *R. coreanus* than in *R. japonicus*.

**Total phenolic, flavanol, flavonoid, and anthocyanin contents in BR fruit samples.** Total phenolic content, flavanol, flavonoid, and anthocyanin contents in the BR samples are summarized in Table 2. Gokseong BR had the highest total phenolic content (1.05 g/100 g dw), Sunchang BR had the highest flavanol content (0.10 g/100 g dw), and Jeongeup BR had the highest flavonoid content (0.19 g/100 g dw). The anthocyanin contents of Gokseong and Hoengseong, which are genetically similar to the native genus (*R. coreanus*), were lower than those of Gochang, Sunchang, Jeongeup, and Jinan. In addition, the flavonoid content of samples from Hoengseong was lower than those of the other BRs.

**Comparison of PLS-DA plots and HCA dendrograms derived from $^{1}$H- NMR spectral data, and total phenolic, flavanol, flavonoid, and anthocyanin contents.** PCA was performed using mean-centered and Pareto-scaled NMR spectral data to identify any outlier and check the separation without prior classification, and the BR samples from each origin clustered in the PCA-derived score plots (supplemental figure 2A, 2B). For clearer separation of each sample and revealing contributing metabolite involved in the separation, PLS-DA was applied to the $^{1}$H-NMR spectral data of BR samples from each origin. PLS-DA score plots

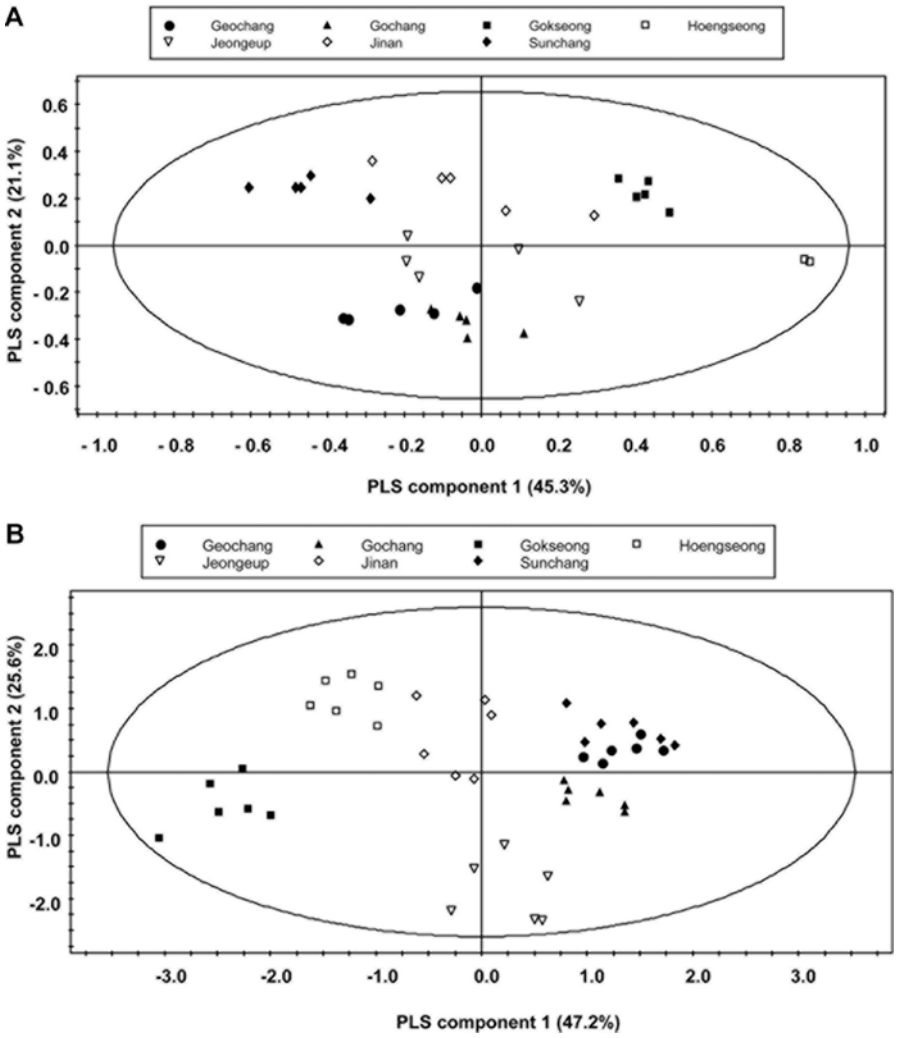


**Fig. 4** PLS-DA score plot derived from $^{1}$H-NMR spectra (A) and total phenolic compounds, flavonol, flavonoid, and anthocyanin content (B) of BR fruit samples of different geographical origins. ●: Geochang BR, ▲: Gochang BR, ◆: Sunchang BR, ▽: Jeongeup BR, ◇: Jinan BR, ■: Gokseong BR, □: Hoengseong

using $^{1}$H-NMR spectral data (a) separated BR samples from Geochang, Gochang, and Sunchang more clearly than plots using total phenolic, flavanol, flavonoid, and anthocyanin contents (b), whereas BR samples from Gokseong and Hoenseong were clearly separated from the other samples in both PLS-DA plots (Fig. 4). These results agreed closely with the RAPD dendrogram. BR fruits from Geochang, Gochang, Sunchang, Jinan, and Jeongeup were regarded as one species, and those from Gokseong and Hoengseong were regarded as different species. These data suggest that BR fruits from Geochang, Gochang, Sunchang, Jinan, and Jeongeup are *R. japonicus*, and those from Gokseong and Hoengseong are *R. coreanus*. Two representative species of BR, *R. coreanus* (Gokseong) and *R. japonicus* (Sunchang) showed different metabolic profiles, even though the cultivation locations were close to each other (Figs. 1 and 4A). Therefore, the difference of original species could greatly influence the metabolic profiles of each BR fruit sample. In addition, the metabolome of BR fruit of the same species might vary according to differences of temperature, rainfalls, sun-exposure time, soil contents, and biological environment of the cultivation locations.

In addition, HCA was used to evaluate the metabolic closeness of each sample. Figure 5 shows dendrograms derived from $^{1}$H-NMR spectral data (A) and total phenolic, flavanol, flavonoid, and anthocyanin contents (B). The dendrogram differentiated two groups. The samples 1, 2, 3, 4, and 5 were grouped together, whereas samples 6 and 8 were grouped separately (Fig. 5A). The HCA dendrogram using the flavonoid, phenolic acid, flavonol, and anthocyanin contents (Fig. 5B) did not match the dendrogram from RAPD genetic fingerprinting (Fig. 2B). However, the dendrogram derived from HCA using NMR-based metabolic fingerprinting data did match the dendrogram from RAPD genetic fingerprinting.

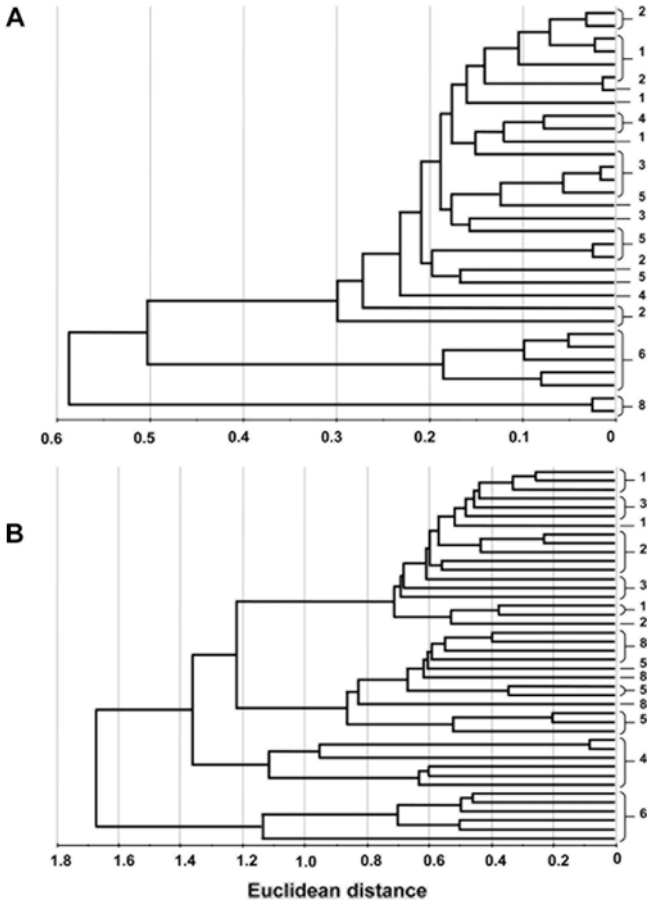


**Fig. 5** HCA dendrograms derived from $^{1}$H-NMR spectral data (A), and total phenolic, flavanol, flavonoid, and anthocyanin contents (B) of BR fruit samples of different geographical origins. 1: Geochang BR, 2: Gochang BR, 3: Sunchang BR, 4: Jeongeup BR, 5: Jinan BR, 6: Gokseong BR, 8: Hoengseong.

Thus, the genomic fingerprinting of leaf samples was shown to match NMR-based metabolic profiling data in the present study. Shoot tips or leaves are considered adequate resources for genomic analysis of BR. Generally, fruits without shoot tips or leaves are available in markets (Graham and McNicol, 1995; Stafne et al., 2003). The main advantage of the present research is that we can differentiate BR fruits of various species and geographical origins using NMR-based metabolic profiling data.

In this study, we showed that $^{1}$H-NMR-based metabolic profiling of BR fruit samples could differentiate and cluster the BR fruit samples according to species and geographic origin. HCA dendrograms derived from $^{1}$H-NMR data grouped fruit samples according to species and geographic origin, and these matched RAPD data of leaf samples, whereas the HCA dendrogram using flavonoid, phenolic acid, flavonol, and anthocyanin contents did not match the RAPD data. Thus, these results suggest that NMR-based metabolic profiling is a useful method to differentiate BR fruit species and geographical origins. To the best of our knowledge, this is the first study matching the genomic and metabolic information of BR samples from different species and geographical origins.

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## References

Abdel-Aal ESM and Hucl P (1999) A rapid method for quantifying total anthocyanins in blue aleurone and purple pericarp wheats. *Cereal Chem* **76**, 350–4.

Anastasiadi M, Zira A, Magiatis P, Haroutounian SA, Skaltsounis AL, and Mikros E (2009) $^{1}$H-NMR-Based Metabonomics for the Classification of Greek Wines According to Variety, Region, and Vintage. Comparison with HPLC Data. *J Agric Food Chem* **57**, 11067–74.

Atak Ç, Alikamanoðlu S, Açık L, and Canbolat Y (2004) Induced of plastid mutations in soybean plant (*Glycine max* L. Merrill) with gamma radiation and determination with RAPD. *Mut Res* **556**, 35–44.

Carroll RJ and Schneider H (1985) A note on levene's tests for equality of variances. *Stat Probab Lett* **3**, 191–4.

de Boer SH, Ward LJ, Li X, and Chittaranjan S (1995) Attenuation of PCR inhibition in the presence of plant compounds by addition of BLOTTO. *Nucleic Acids Res* **23**, 2567.

de Ancos B, Gonzalez EM, and Cano MP (2000) Ellagic acid, vitamin C, and total phenolic contents and radical scavenging capacity affected by freezing and frozen storage in raspberry fruit. *J Agric Food Chem* **48,** 4565–70.

Eriksson L, Johansson E, Kettaneh-Wold N, Wold S, Trygg J, and Wikström C (2006) In *Multi- and Megavariate Data Analysis Part I: Basic Principles and Applications,* (2nd ed.). Umetric Academy, Sweden.

Fiehn O, Kopka J, Dörmann P, Altmann T, Trethewey RN, and Willmitzer L (2000) Metabolite profiling for plant functional genomics. *Nat Biotechnol* **18**, 1157–61.

Gajera BB, Kumar N, Singh AS, Punvar BS, Ravikiran R, Subhash N et al. (2010) Assessment of genetic diversity in castor (*Ricinus communis* L.) using RAPD and ISSR markers. *Ind Crop Prod* **32**, 491–8.

Graham J and McNicol RJ (1995) An examination of the ability of RAPD markers to determine the relationships within and between *Rubus* species. *Theor Appl Genet* **90**, 1128–32.

Hamada K and Hagimori M (1996) RAPD-based method for cultivar-identification of calla lily (*Zantedeschia spp.*). *Sci Hortic* **65**, 215–8.

Hartigan JA (1985) Statistical theory in clustering. *J Classif* **2**, 63–76.

John ME (1992) An efficient method for isolation of RNA and DNA from plants containing polyphenolics. *Nucleic Acids Res* **20**, 2381.

Jung Y, Lee J, Kwon J, Lee KS, Ryu DH, and Hwang GS (2010) Discrimination of the Geographical Origin of Beef by $^{1}$H-NMR-Based Metabolomics. *J Agric Food Chem* **58**, 10458–66.

Kim HC and Lee SI (1991) Comparision of the pharmacological effect of kinds *Rubi fructus*. *J Herbology* **6,** 3–12.

Kim HS, Park SJ, Hyun SH, Yang SO, Lee J, Auh JH et al. (2011) Biochemical monitoring of black raspberry (*Rubus coreanus* Miquel) fruits according to maturation stage by $^{1}$H-NMR using multiple solvent systems. *Food Res Int* **44**, 1977–87.

Kim KH, Lee YA, Kim JS, Lee DI, Choi YW, Kim HH et al. (2000) Antioxidative activity of tannins from *Rubus coreanum*. *Yakhak Hoeji* **44**, 354–7.

Kim MS, Pang GC, and Lee MW (1997) Flavonoids from the leaves of *Rubus coreanum*. *Yakhak Hoeji* **41,** 1–6.

Kwak J, Park S, and Yoon MK (2009) Application of RAPD and SI-typing techniques to confirm genetic integrity of radish (*Raphanus sativus* L.)

varieties. *Sci Hortic* **119**, 352–6.

Kwon MC, Kim CH, Na CS, Kwak HG, Kim JC, and Lee HY (2007) Comparison of Immuno-Modulatory Regulatory Activities of *Rubus coreanus* Miquel by Ultra High Pressure Extracts Process. *Korean J Medicinal Crop Sci* **15,** 398–404.

Lee JE, Lee BJ, Chung JO, Hwang JA, Lee SJ, Lee CH et al. (2010) Geographical and climatic dependencies of green tea (*Camellia sinensis*) metabolites: A $^{1}$H-NMR-based metabolomics study. *J Agric Food Chem* **58**, 10582–9.

Lee JW and Do JH (2000) Determination of Total Phenolic Compounds from the Fruit of *Rubus coreanum* and Antioxidative Activity. *J Korean Soc Food Sci Nutr* **29,** 943.

Lee Y, Yang VC, and Wang T (2007) Use of RAPD to detect sodium arsenite-induced DNA damage in human lymphoblastoid cells. *Toxicology* **239**, 108–15.

Longobardi F, Ventrella A, Napoli C, Humpfer E, Schütz B, Schäfer H et al. (2012) Classification of olive oils according to geographical origin by using $^{1}$H NMR fingerprinting combined with multivariate analysis. *Food Chem* **130**, 177–83.

Pasqualone A, Montemurro C, Caponio F, and Blanco A (2004) Identification of virgin olive oil from different cultivars by analysis of DNA microsatellites. *J Agric Food Chem* **52**, 1068–71.

Porter LJ, Hrstich LN, and Chan BG (1985) The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* **25**, 223–30.

Quettier-Deleu C, Gressier B, Vasseur J, Dine T, Brunet C, Luyckx M et al. (2000) Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. *J Ethnopharmacol* **72**, 35–42.

Singleton VL and Rossi Jr JA (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic* **16**, 144–58.

Stafne ET, Clark JR, Pelto MC, and Lindstrom JT (2003) Discrimination of *Rubus* cultivars using RAPD markers and pedigree analysis. *Acta Hort* **626**, 119–24.

Tian Q, Giusti MM, Stoner GD, and Schwartz SJ (2006) Characterization of a new anthocyanin in black raspberries (*Rubus occidentalis*) by liquid chromatography electrospray ionization tandem mass spectrometry. *Food Chem* **94**, 465–8.

Wyzgoski FJ, Paudel L, Rinaldi PL, Reese RN, Ozgen M, Tulio Jr AZ et al. (2010) Modeling relationships among active components in black raspberry (*Rubus occidentalis* L.) fruit extracts using high-resolution $^{1}$H-nuclear magnetic resonance (NMR) spectroscopy and multivariate statistical analysis. *J Agric Food Chem* **58**, 3407–14.

Yoo NH, Baek SH, and Yun SJ (2001) A simple and reliable method for PCR-based analyses in plant species containing high amounts of polyphenols. *Korean J Plant Res* **14**, 235–40.

Yoon I, Wee JH, Moon JH, Ahn TH, and Park KH (2003) Isolation and identification of quercetin with antioxidative activity from the fruits of *Rubus coreanum* Miquel. *Korean J Food Sci Technol* **35**, 499–502.

Zhishen J, Mengcheng T, and Jianming W (1999) The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem* **64**, 555–9.