Conclusion

In this study, we developed a trait-specific method that can quantitate GMO content by measuring only *cp4epsps* and *pat* using qPCR. This method overcomes the drawback associated with event-specific methods, in which the GMO content of stacked GM maize samples is greatly overestimated. The developed trait-specific method would be the only feasible way to conduct the quantification of GMO content in the ground maize samples containing stacked GM maize, which will increasingly be found in the future, for the verification of the labeling regulation.

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Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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ORIGINAL PAPER

Genetic and chemical characterization of white and red peony root derived from *Paeonia lactiflora*

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Abstract Two kinds of peony roots—white peony root (WPR) and red peony root (RPR)—are used for different remedies in traditional Chinese medicine; however, most of them are derived from the same botanical origin, *Paeonia lactiflora*. The difference between WPR and RPR has been debated for a long time. This study attempted to clarify the genetic and chemical characteristics of WPR and RPR in order to provide a scientific dataset for their identification and effective use. The nucleotide sequence of nrDNA internal transcribed spacer (ITS) and the contents of 8 main bioactive constituents were analyzed from specimens of *P. lactiflora*, *P. veitchii* and two related species as well as crude drug samples of WPR, RPR and peony root produced in Japan. Of the samples derived from *P. lactiflora*, the WPR produced in the southern parts of China and the RPR produced in the northern parts of China were clearly divided into two subgroups within the *P. lactiflora* group based on similarity of the ITS sequences. The nucleotides at positions 69, 458 and 523 upstream of the ITS sequence served as molecular markers to discriminate between WPR and RPR. Quantitative analysis indicated that the RPR samples obviously contained a higher content of paeoniflorin and paeonol, but a lower content of albiflorin than the WPR produced in the southern parts of China and peony root produced in Japan. The WPR available from Chinese markets was usually processed by sulfur fumigation, which resulted in an extremely low content of paeoniflorin. This study indicated that WPR and RPR were not only geographically isolated, but also genetically and chemically separated. The ITS sequence provided a genetic index for their identification.

Keywords *Paeonia lactiflora* · White peony root · Red peony root · Internal transcribed spacer of nrDNA (ITS) sequence · Chemical composition · Paeoniflorin · Pentagalloylglucose

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Introduction

Peony root (PR) (Paeoniae Radix) is one of the most frequently used crude drugs in traditional Chinese medicine and Kampo medicine. In China there are two kinds of PR available—white peony root (WPR) and red peony root (RPR)—which are used for different remedies. WPR has been used to treat symptoms such as dizziness, limb spasm, abdominal pain, diarrhea, blood deficiency and irregular menses, etc. RPR, however, has been mainly used as remedies for blood stasis, gynecological diseases, cardiovascular illness, etc. [1]. In Chinese Pharmacopoeia, WPR is prescribed as the root of *Paeonia lactiflora* Pallas, which has been boiled and peeled and RPR is prescribed as the naturally dried root of *P. lactiflora* and *P. veitchii* Lynch. With the exception of *P. veitchii*-derived RPR, the WPR and RPR are from the same botanical origin, *P. lactiflora*. The difference between WPR and RPR has been debated for a long time with regard to the processing methods (peeled or unpeeled), difference in flower colors or root

colors, and cultivated or wild, etc. [2]. The most recent Pharmacognosy textbook describes that WPR is derived from cultivated sources mainly produced in Anhui, Zhejiang, and Sichuan provinces of southern China, whereas RPR is derived from wild plants collected from Inner Mongolia and northeastern China [3]. It is interesting to explore the underlying differences between these two crude drugs. It is also an appealing yet difficult task in terms of how to accurately discriminate between them. In this study, we attempted to find the answers using genetic and chemical approaches.

In the Japanese Pharmacopoeia, PR is prescribed as the root of *P. lactiflora* with not less than 2.0 % paeoniflorin [4]. As an analgesic, antispasmodic, astringent, etc., it has been included in approximately one-third of Kampo formulas used in Japan [5]. Hereafter, PR refers limitedly to the crude drugs of the roots of *P. lactiflora* available from Japanese markets. PR is supplied mainly by import from China and partly by domestic production, e.g., 1,188 tons was imported in 2010 and the domestic supply was 38 tons [6]. Apart from the different processing methods, PR and WPR are considered to be almost the same crude drug, because both are derived from the cultivated *P. lactiflora*. Generally, the PR samples imported from China are produced in the same producing areas of WPR.

To date, genetic markers used in phylogenetic analysis of *Paeonia* species include nucleotide sequences of the ribosomal DNA internal transcribed spacer (ITS) region, Adh1/2 genes and GPAT gene of nDNA, as well as *mat*K gene, intergenic spacer *trn*L-F and *psb*A–*trn*H of cpDNA [7–10]. Among them, the ITS sequence has been demonstrated to be powerful for phylogenetic resolution and for discrimination between species due to the high rate of variation [11, 12]. Moreover, ITS sequencing of *Paeonia* species provides the most successful example of using the ITS sequence to reconstruct reticulate evolution; the nucleotide additivity is highly informative for detecting hybridization and inferring parental lineages [7]. Phytochemical studies have indicated that *Paeonia* plants contain many bioactive components, including monoterpenes, flavonoids, phenols and tannins [13]. Several reports have described quantitative analyses of the main chemical components of commercial samples of WPR and RPR [14, 15].

The present study determined the nucleotide sequence of the ITS region and conducted quantitative analysis of 8 main bioactive components on 4 *Paeonia* species, as well as commercial samples of PR, WPR and RPR collected from Japanese and Chinese markets to clarify the genetic and chemical characteristics of WPR and RPR.

Table 1 Plant specimens used in this study

Original plant	Location for collecting plant	W/C	Voucher no.	Code no.	Collection date
Paeonia lactiflora Pallas	Duolun, Inner Mongolia, China	W	Wei S.L., 2007-1	P1[a]	2007.01.28
P. lactiflora	Arxan, Inner Mongolia, China	W	Murakami M. MM-1	P2	2007.08.11
P. lactiflora	Chifeng, Inner Mongolia, China	W	Komatsu K. et al., HJN269	P14	2012.08.04
P. lactiflora	Wudalianchi, Heilongjiang, China	W	Komatsu K. et al., HJN143	P15	2012.07.22
P. lactiflora	Wudalianchi, Heilongjiang, China	W	Komatsu K. et al., HJN145	P16	2012.07.22
P. lactiflora	Mongolia	W	623-63	P3	1967
P. lactiflora	Xinwo, Zhejiang, China	C	Komatsu K. et al., CJZ132	P9[a]	2009.08.05
P. lactiflora (Bonten)	Toyama, Japan	C	Murakami M., S34	S34[a]	2008.11.
P. lactiflora (Kitasaisho)	Toyama, Japan	C	Murakami M., S31	S31[a]	2008.11.
P. anomala Linn.	Tuluugiyn davas-Hutag-Ondor, Bulgan, Mongolia	W	Komatsu K. et al., M505	P4[a]	2002.07.20
P. anomala	Jingiin halzan-Bayannuul, Uvs, Mongolia	W	Komatsu K. et al., M755-1	P5[a]	2002.07.29
P. anomala	Mongolia	W	Komatsu K. et al., M755-2	P6[a]	2002.07.29
P. veitchii Lynch	Ganzi, Sichuan, China	W	Komatsu K. et al., S-1	P7[a]	1996.07.10
P. japonica Miyabe et Takeda	Toyama, Japan	C	Murakami M., MM-4	P8	2008.12.04

W, wild; C, cultivated

[a] Samples used in chemical analysis

Table 2 Crude drug samples used in this study

Drug name	Abr.	Production area	Obtained from	Code no.	Collection date	TMPW no.
White peony root	WPR	Bozhou, Anhui, China	Bozhou, Anhui, China	D1[a]	2006.05.02	25071
White peony root	WPR	Bozhou, Anhui, China	Bozhou, Anhui, China	D2[a]	2006.05.02	25072
White peony root	WPR	Bozhou, Anhui, China	Bozhou, Anhui, China	D3[a]	2006.10.17	25244
White peony root	WPR	Bozhou, Anhui, China	Bozhou, Anhui, China	D17[a]	2006.05.02	25073
White peony root	WPR	Anhui, China	Hohhot, Inner Mongolia, China	*D23*[a]	2010.10.14	26975
White peony root	WPR	Zhongjiang, Sichuan, China	Jihuang, Zhongjiang, Sichuan, China	D4[a]	2002.09.23	25820
White peony root	WPR	Zhejiang, China	Cenxi, Guangxi, China	D18[a]	2008.09.06	25973
White peony root	WPR	Hangzhou, Zhejiang, China	Hohhot, Inner Mongolia, China	D22[a]	2010.10.13	26974
White peony root	WPR	Pan'an, Zhejiang, China	Pan'an, Zhejiang, China	D29[a]	2009.08.05	26620
White peony root	WPR	Jiangsu, China	Hohhot, Inner Mongolia, China	D24[a]	2010.10.16	26976
Peony root	PR	Anhui, China	National Institute of Biomedical Innovation, Japan	D30	2010	27685
Peony root	PR	Anhui, China	National Institute of Biomedical Innovation, Japan	D31	2009	27689
Peony root	PR	Anhui, China	National Institute of Biomedical Innovation, Japan	D32	2008	27690
Peony root	PR	Anhui, China	National Institute of Biomedical Innovation, Japan	D33	2008	27691
Peony root	PR	Anhui, China	National Institute of Biomedical Innovation, Japan	D34	2009	27693
Peony root	PR	Anhui, China	National Institute of Biomedical Innovation, Japan	D35	2010	27696
Peony root	PR	Anhui, China	National Institute of Biomedical Innovation, Japan	D36	2009	27697
Peony root	PR	Zhejiang, China	National Institute of Biomedical Innovation, Japan	D37	2010	27686
Peony root	PR	Sichuan, China	National Institute of Biomedical Innovation, Japan	D38	2009	27687
Peony root	PR	Sichuan, China	National Institute of Biomedical Innovation, Japan	D39	2009	27692
Peony root	PR	Sichuan, China	National Institute of Biomedical Innovation, Japan	D40	Unknown	27699
Peony root	PR	Zhejiang, China	Tochimoto Tenkaido Co., Ltd., Osaka, Japan	*D45*[a]	2012.09.04	27887
Peony root	PR	Sichuan, China	Tochimoto Tenkaido Co., Ltd., Osaka, Japan	*D46*[a]	2012.09.04	27888
Peony root	PR	Anhui, China	Tochimoto Tenkaido Co., Ltd., Osaka, Japan	*D47*[a]	2012.09.04	27889
Peony root	PR	China	Uchida Wakanyaku Co., Ltd., Tokyo, Japan	*D48*[a]	2012.09.04	27890
Peony root	PR	China	Matsuura Co., Ltd., Nagoya, Japan	D53[a]	2012.10.04	27892
Peony root (Bonten)	PR	Toyama, Japan	Nara, Japan	D50[a]	2008.02.15	25834
Peony root (Bonten)	PR	Toyama, Japan	Nara, Japan	D51[a]	2008.02.15	25835
Peony root (Bonten)	PR	Toyama, Japan	Nara, Japan	D52[a]	2008.02.15	25836
Peony root	PR	Niigata, Japan	Uchida Wakanyaku Co., Ltd., Tokyo, Japan	*D6*[a]	2008.10.07	26400
Peony root (Yamato)	PR	Nara, Japan	Tochimoto Tenkaido Co., Ltd., Osaka, Japan	D7[a]	2008.02.14	25818
Peony root (Yamato)	PR	Hokkaido and Nagano, Japan	Tochimoto Tenkaido Co., Ltd., Osaka, Japan	D8[a]	2008.02.14	25819
Peony root (Yamato)	PR	Nara, Japan	Tochimoto Tenkaido Co., Ltd., Osaka, Japan	D9[a]	2008.10.31	26107

Table 2 continued

Drug name	Abr.	Production area	Obtained from	Code no.	Collection date	TMPW no.
Peony root (Yamato)	PR	Nara, Japan	Tochimoto Tenkaido Co., Ltd., Osaka, Japan	D10[a]	2008.10.31	26398
Peony root	PR	Nagano, Japan	National Institute of Biomedical Innovation, Japan	D41	2009	27688
Peony root	PR	Nara, Japan	National Institute of Biomedical Innovation, Japan	D42	2010	27694
Peony root	PR	Niigata, Japan	National Institute of Biomedical Innovation, Japan	D43	2010	27695
Peony root	PR	Nara, Japan	National Institute of Biomedical Innovation, Japan	D44	2010	27698
Peony root	PR	Japan	Uchida Wakanyaku Co., Ltd., Tokyo, Japan	D49[a]	2012.09.04	27891
Red peony root	RPR	Sichuan, China	Cenxi, Guangxi, China	D16[a]	2008.09.06	25974
Red peony root	RPR	Inner Mongolia, China	Chifeng, Inner Mongolia, China	D12[a]	2002.09.14	21565
Red peony root	RPR	Inner Mongolia, China	Bozhou, Anhui, China	D13[a]	2006.05.01	25047
Red peony root	RPR	China	Uchida Wakanyaku Co., Ltd., Tokyo, Japan	*D14*[a]	2008.10.07	26401
Red peony root	RPR	Inner Mongolia, China	Tochimoto Tenkaido Co., Ltd., Osaka, Japan	D15[a]	2008.10.16	26406
Red peony root	RPR	China	Matsuura Co., Ltd., Nagoya, Japan	D54[a]	2012.10.04	27893
Red peony root[b]	RPR[b]	Ganzi, Sichuan, China	Ganzi, Sichuan, China	D11[a]	1996.07.11	17304

[a] Samples used in chemical analysis; italicized number: multiple individuals in the sample were analyzed

[b] This sample is derived from *P. veitchii*; apart from this sample the others are derived from *P. lactiflora* based on sequence analysis results

Materials and methods

Plant and crude drug materials

Fourteen specimens of four *Paeonia* species were analyzed—*P. lactiflora*, *P. veitchii*, *P. anomala* Linn. and *P. japonica* Miyabe et Takeda. Details of the plant materials are shown in Table 1. Meanwhile, 46 commercial samples available from Chinese and Japanese markets were collected and analyzed for comparison. Of the PR samples collected from Japanese markets, half were imported from China and half were produced in Japan. In several commercial samples, multiple individuals were analyzed and their identities were indicated separately. All of the plant specimens and crude drug samples were stored in the Museum of Materia Medica, Institute of Natural Medicine, University of Toyama, Japan (TMPW) (Table 2).

Genomic DNA extraction and PCR amplification

Total DNA was extracted from 50 − 60 mg of root powder by using DNeasy™ Plant Mini Kit (Qiagen, Germany) with little modification to the protocol provided by the manufacturer. The primer pair used for PCR amplification were ITS-1F (5′-GTA GGT GAA CCT GCA GAA GGA TCA-3′) and 18S-25S-3′R (5′-CCA TGC TTA AAC TCA GCG GGT-3′) as reported previously [16], which was able to amplify a fragment of approximately 700 bp including the rDNA ITS1–5.8S–ITS2 regions. PCR amplification was performed in 25 μl of reaction mixture, consisting of 1 × PCR buffer for KOD-Plus-, 0.2 mM of each dNTP, 1.0 mM $MgSO_4$, 0.3 μM of each primer, 0.5 U KOD-Plus-DNA polymerase (Toyobo, Japan), and 0.5 μl of total DNA. PCR amplification was performed under the cycling profile of a preliminary denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 50 s, and a final extension at 68 °C for 10 min by a Takara thermal cycler (Takara, Japan). PCR products were purified with a WizardSV PCR Clean-Up System (Promega, USA).

Sequence analysis and clustering analysis

Sequencing reaction of purified PCR products was carried out separately using ABI PRISM Bigdye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, USA) with each of the 4 types of primers (ITS-1F, 18S-25S-3′R, In-18S-25S-5′F: 5′-TCT CGC ATC GAT GAA GAA CG-3′ and In-18S-25S-3′R: 5′-GAC TCG ATG GTT CAC GGG ATT CT-3′). The sequence was determined directly using ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, USA). With regard to the judgment of additive sites, we calculated the relative intensity of the lower peak as *S* value (lower peak intensity/sum of main peak intensity and lower peak intensity) for the sites where double peaks occurred. The sites with an *S* value no less than 15 % were recorded as additive sites, according to our previous results

Table 3 Comparison of ITS sequences among various samples derived from *Paeonia lactiflora*

Samples	ITS-1												5.8S	ITS-2							
	4 6	6 5	6 9	9 3	1 2 5	1 3 5	2 1 2	2 1 4	2 1 7	2 2 6	2 3 9	2 4 6	3 9 7	4 4 ~ 3 8		4 5 8	4 6 8	5 2 3	5 9 5	6 1 8	6 2 2	Acces.No. in INSD
Paeonia lactiflora (type 1)	G	G	C	G	C	C	G	C	C	A	G	G	T	CCCCCC		C	A	C	G	C	G	U27682
P. lactiflora (type 2)	*	*	T	*	*	*	*	*	*	*	*	*	*	******_		A	*	T	*	*	*	JN572150 FJ514503
D1 WPR	*	R	T	*	*	*	*	Y	Y	*	R	K	*	******_		A	*	T	*	*	*	AB934995
D33 PR	*	K	T	*	Y	*	*	*	*	*	R	K	*	******_		A	*	T	*	*	*	AB934996
P9	*	K	T	*	Y	*	*	*	Y	*	R	K	*	******_		A	*	T	*	*	*	AB934997
D10 PR, D44 PR, D46-2 PR, D52 PR, *S31*, *S34*,	*	*	T	*	Y	*	*	*	Y	*	R	K	*	******_		A	*	T	*	*	*	AB934998
D40 PR	*	*	T	*	Y	*	*	*	Y	*	R	K	*	******_		A	*	T	*	Y	K	AB934999
D3 WPR, D37 PR, D38PR	*	*	T	*	Y	*	*	*	Y	*	R	K	*	******_		A	*	T	*	Y	*	AB935000
D4 WPR, D7 PR	*	*	T	*	*	*	*	*	Y	*	R	K	*	******_		A	*	T	*	Y	*	AB935001
D30 PRc,	*	K	T	*	*	*	*	*	Y	*	R	K	*	*****Y-		A	*	T	*	Y	K	AB935002
D39-2 PR	*	K	T	*	*	*	*	*	Y	*	R	K	*	*****Y-		A	*	T	*	*	K	AB935003
D17 WPR, D23-1 WPR, D35 PR, D39-1 PR, D47-2 PR, D53-2 PR	*	*	T	*	*	*	*	*	Y	*	R	K	*	*****Y-		A	*	T	*	Y	K	AB935004
D2 WPR, D31 PR, D34 PR, D45-2 PR, D46-1 PR, D48-1,2 PR	*	K	T	*	Y	*	*	*	Y	*	R	K	*	*****Y-		A	*	T	*	Y	K	AB935005
D32 PR, D36 PR, D16 RPR	*	*	T	*	Y	*	*	*	Y	*	R	K	*	*****Y-		A	*	T	*	Y	K	AB935006
D18 WPR	*	K	T	*	*	*	*	*	Y	*	R	K	*	*****Y-		A	*	T	*	*	*	AB935007
D23-2 WPR	*	*	T	*	*	*	*	*	Y	*	R	K	*	*****Y-		A	*	T	*	*	*	AB935008
D8 PR	*	*	T	*	Y	*	*	*	Y	*	R	K	*	******_		M	*	T	*	*	*	AB935009
D6-2 PR, D6-5 PR	*	*	T	*	Y	*	*	*	Y	*	R	K	*	******_		M	*	Y	*	Y	*	AB935010
P3	*	*	Y	R	Y	*	R	*	Y	R	*	*	*	******_		M	W	Y	R	*	*	AB935011
D14-4 RPR	*	*	Y	*	Y	*	R	*	Y	R	*	*	Y	******_		M	W	Y	*	*	*	AB935012
D14-3 RPR	*	*	Y	R	Y	*	R	*	Y	R	*	*	Y	******_		M	W	Y	*	*	*	AB935013
P14, P16, **D14-1 RPR**	R	*	Y	R	Y	*	R	*	Y	R	*	*	Y	******_		M	W	Y	*	*	K	AB935014
D13 RPR	R	*	Y	R	Y	*	R	*	Y	*	*	*	Y	******_		M	W	Y	*	*	K	AB935015
D42 PR, D54-1 RPR	R	*	Y	R	Y	*	*	*	Y	*	*	*	Y	******_		M	W	Y	*	*	K	AB935016
D54-2 RPR	R	*	Y	R	Y	*	R	*	Y	*	*	*	Y	******_		M	W	Y	R	*	K	AB935017
P15, **D14-2,5 RPR**	R	*	Y	R	*	*	R	*	Y	*	*	*	Y	******_		M	W	Y	*	*	K	AB935018
D12 RPR	R	S	Y	R	Y	Y	R	*	Y	*	*	*	Y	******_		M	W	Y	*	*	K	AB935019
D6-1 PR, D6-4 PR	R	*	Y	R	*	*	*	*	Y	*	R	K	*	******_		M	*	Y	*	*	K	AB935020
D49-2 PR	R	*	Y	R	Y	*	*	*	Y	*	R	K	*	******_		M	*	Y	*	*	K	AB935021
D9 PR, D49-1 PR	R	*	Y	R	Y	*	*	*	Y		R	*	*	******_		M	*	Y	*	*	K	AB935022
P2, D41 PR, D43 PR	R	*	Y	R	Y	*	*	*	Y	*	*	*	*	******_		M	*	Y	*	*	K	AB935023
D6-3 PR	R	*	Y	R	Y	*	*	*	*	*	*	*	*	******_		M	*	Y	*	*	K	AB935024
D15 RPR	R	S	Y	R	*	Y	R	*	Y	R	*	*	*	******_		M	*	Y	*	*	K	AB935025
P1	R	S	Y	R	*	Y	*	*	Y	*	*	*	*	******_		M	*	Y	*	*	K	AB935026

K = G&T; M = A&C; R = A&G; S = C&G; W = A&T; Y = C&T

Italic numbers, plant materials; bold faced number, RPR produced in China; underlined numbers, PR produced in Japan

from experimental examinations [17, 18]. The borders of ITS1, 5.8S, and ITS2 regions were determined by comparison with the known sequences of mung bean and rice [19, 20]. The nucleotides sequence data of the ITS1–5.8S–ITS2 regions were deposited in the International Nucleotide Sequence Database (INSD: DDBJ/EMBI/NCBI) with the accession numbers shown in Table 3.

Comparison of ITS sequences was carried out by the ClastalW2 program for multiple sequence alignment. A guide tree on the basis of sequence similarity was constructed during multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Besides the sequences determined in the present study, ITS sequences of related *Paeonia* species obtained from INSD were also included.

HPLC analysis of 8 main components

Quantitative analysis of 8 main components (paeoniflorin, albiflorin, pentagalloylglucose [PGG], (+)-catechin, gallic acid, methyl gallate, benzoic acid and paeonol) (Fig. 1) was conducted using the reported method [14, 21] with little modification. Of the standards, paeoniflorin, albiflorin and paeonol were purchased from Wako Pure Chemical Industries (Osaka, Japan), PGG from Toronto Research Chemicals (ON, Canada), (+)-catechin from Cayman Chemical Company (MI, USA), gallic acid and benzoic acid from Nacalai Tesque Inc. (Japan), and methyl gallate from ChromaDex (CA, USA). Reagents for HPLC analysis including acetonitrile, distilled water (both of HPLC grade) and phosphatic acid (analytical grade) were purchased from Wako Pure Chemical Industries (Japan).

A Jasco HPLC system equipped with a PU-1580 pump, an LC-1580-02 ternary gradient unit, and an MD-1510 multiple wavelength detector was used. Analysis was carried out using a YMC Pack AQ-303 (ODS, 4.6 mm i.d. × 250 mm, 5 μm) with column temperature at 27 °C. The mobile phase consisted of binary eluents of (A) 0.1 % (v/v) phosphatic acid and (B) CH_3CN under gradient conditions (0 min, 10 % B; 5 min, 15 % B; 40 min, 30 % B; 45 min, 70 % B; 46 min, 80 % B; 50 min, 80 % B; 55 min, 10 % B; 65 min, 10 % B). Flow rate was 1.0 ml/min. Detection was performed at a wavelength of 232 nm.

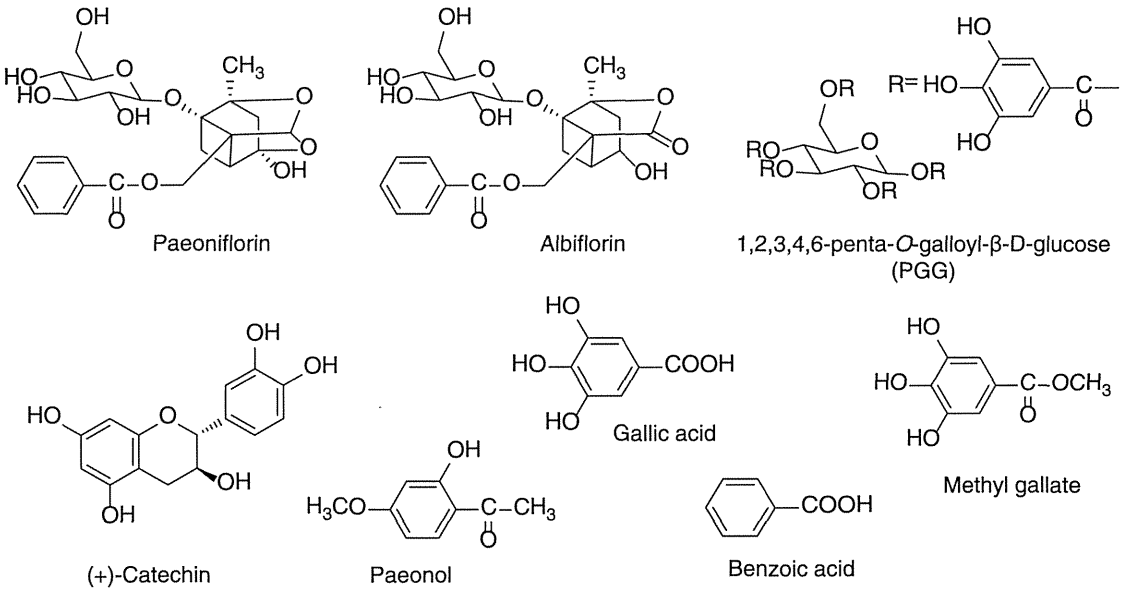

Fig. 1 Chemical structures of 8 compounds

Each standard was accurately weighed and dissolved in 75 % EtOH to make a stock solution of 1.0 mg/ml. To make calibration curves, a series of standard solutions (200, 100, 20, 10, 2 μg/ml) were prepared from the stock solution. The calibration curve of each component was prepared by plotting the peak areas against a series of injection amounts.

The respective samples (10–50 g) were pulverized and then screened through a 300-μm sieve to obtain homogeneous fine powder of each sample; 0.3 g of the fine powder was accurately weighed and extracted with 75 % EtOH (9 ml, 8 ml × 2) by ultrasonication at room temperature for 30 min, mixed periodically by vortex to obtain full extraction. The supernatant was then obtained by centrifugation at 2,500 rpm (Kubota 3740, Japan) for 10 min. Supernatants were combined into a 25.0-ml volumetric flask and finally filled with 75 % EtOH to the volume. After filtration through a 0.2-μm Millipore filter unit (Advantec, Japan), 20.0 μl of this solution was injected into the HPLC system for analysis.

The quantitative analysis data was further subjected to principal component analysis (PCA) by using software IBM SPSS Statistics (Version 19.0) to facilitate characterization of WPR and RPR.

Results and discussion

Genetic characterization based on ITS sequences

The ITS sequences of specimens of the four *Paeonia* species as well as the crude drug samples were clearly determined and compared. All the sequences were of the same length, in which the ITS1 region was 267 bp, the 5.8S rRNA gene region was 164 bp, and the ITS2 region was 221 bp. The specimens of the 4 species showed their own ITS sequences which had > 99 % homology to the corresponding sequences of the respective species registered in INSD. All the determined sequences were compared using ClastalW2, a program for multiple sequence alignment. The program constructed a guide tree (Fig. 2) on the basis of sequence similarity to facilitate the alignment. In this guide tree, *P. lactiflora* formed a large group which was completely separate from *P. veitchii*, *P. anomala*, *P. japonica* and *P. suffruticosa*. Of the crude drug samples collected from markets, only a sample of RPR (D11) collected from Sichuan province presented an almost identical sequence to that of *P. veitchii* (P7) and was identified as *P. veitchii* (Fig. 2). Other samples which included both WPR and RPR were confirmed to be *P. lactiflora* based on the sequence similarity. Within the group of *P. lactiflora*, two main subgroups were further clustered (Fig. 2). It is noteworthy that the WPR produced in the southern parts of China belonged to one subgroup (WPR subgroup) and the RPR produced in the northern parts of China fell into another subgroup (RPR subgroup) (Fig. 2). The results clearly indicated that the ITS sequence was informative and sufficiently powerful to discriminate *Paeonia* plants at either inter-species or intra-species levels and suggested that WPR and RPR were geographically isolated and genetically separated.

Within *P. lactiflora*, significant intra-species polymorphism of the ITS sequences was detected. Although Sang et al. [7] reported that there was almost no polymorphism

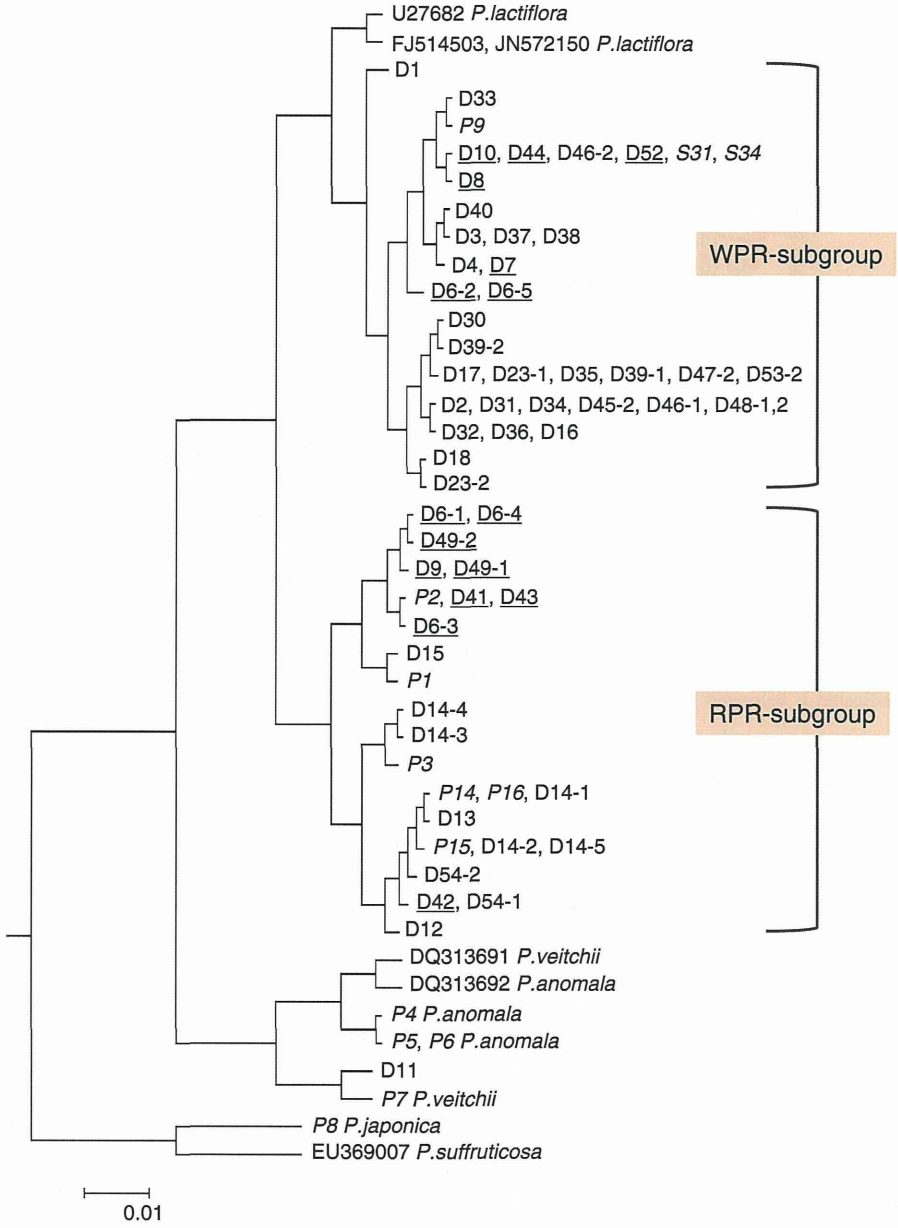

Fig. 2 Guide tree obtained from multiple sequence alignment on the basis of sequence similarity. *Italic numbers, plant materials; underlined numbers, PR produced in Japan

in the ITS sequence among intraspecific populations, the present study, by sampling dozens of accessions widely collected from China and Japan, indicated a significant polymorphism in the ITS sequences of *P. lactiflora*. Table 3 shows nucleotide differences among various types of ITS sequences from *P. lactiflora*-derived samples. The ITS sequences of *P. lactiflora* deposited in INSD included two main types, which differed from each other by three nucleotides at positions 69, 458 and 523 (Table 3). Type 1 (U27682 reported by Sang et al. [7]) shows three cytosines (C–C–C), while type 2 (JN572150 reported by Sun and Hong [22]) has thymine, adenosine and thymine (T-A-T) at the three sites. According to the nucleotides at these three sites, the samples analyzed in the present study were divided into two main groups corresponding to the two subgroups as shown in Fig. 2. One subgroup which included all the samples of WPR produced in Anhui, Zhejiang and Sichuan provinces and the two Japanese medicinal cultivars (S31, S34) showed T_{69}-A_{458}-T_{523} at the same three sites as the type 2. The other subgroup which included plant specimens of wild *P. lactiflora* (P1–P3) as well as the RPR samples, except for sample D16 from Sichuan province, showed additive nucleotides (double nucleotides detected at the same site) as Y (T&C), M (A&C) and Y at the three sites (Y_{69}-M_{458}-Y_{523}), respectively. Three PR samples (D6-2, D6-5, D8) with additive nucleotides at one or two of the three sites presented intermediate type of sequences.

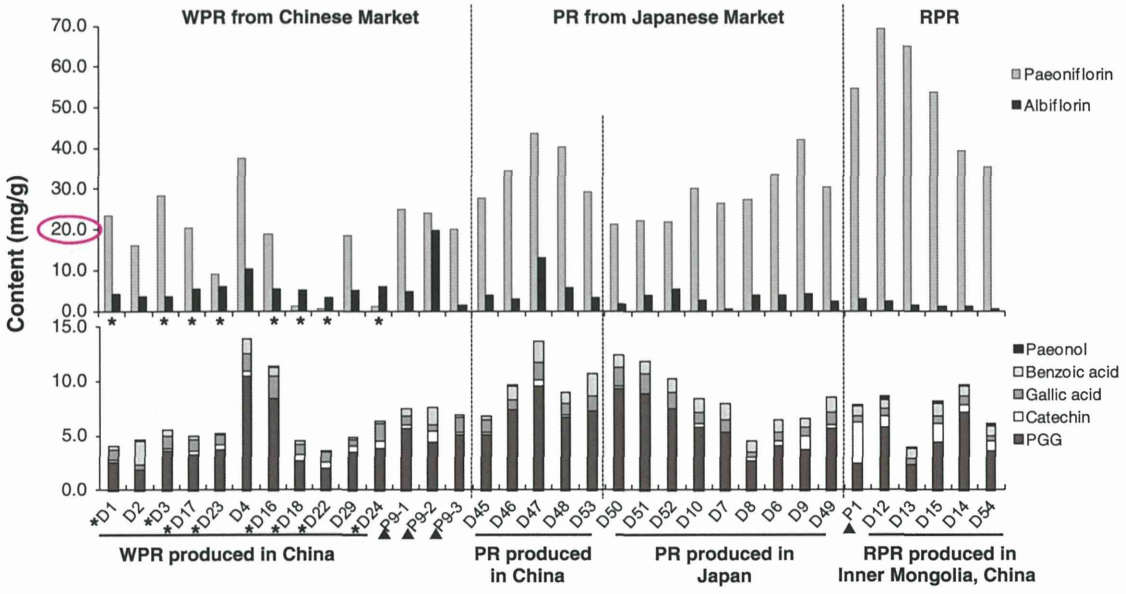

Fig. 3 Contents of 8 compounds in WPR, PR and RPR derived from *P. lactiflora*. *WPR which was processed by sulfur fumigation. D16 was purchased in Guangxi, China as RPR; however, genetic analysis indicated it belonged to the WPR subgroup. Therefore we placed it within the WPR group. ▲P9 and P1 were collected in Zhejiang and Inner Mongolia, respectively, where are the production areas of WPR and RPR

Apart from the difference at the above three sites, nucleotide additivities were observed at another 17 positions (Table 3). Due to the bi-parental inheritance mode of the nrDNA ITS region, nucleotide additivities detected in the sequence are potentially advantageous features for speculating hybridization and inferring progenitors and lineages [7]. However, lineages other than *P. lactiflora* which are possibly involved and responsible for these nucleotide additivities could not be implied even through wide comparison of the ITS sequences from genus *Paeonia* registered in INSD. Future investigation around wild habitats of *P. lactiflora* and subsequent sequencing of related samples might provide further evidence. Among such sites, additivities detected at nucleotide positions 239 and 246 were found to be in common within the WPR subgroup, and those at position 93 were almost in common within the RPR subgroup. Detailed sequence comparison indicated that the divergent sites in the ITS sequence have the potential to discriminate between WPR and RPR; in particular, the nucleotides at positions 69, 458 and 523 were the most informative.

The two Japanese medicinal cultivars of *P. lactiflora*—'Bonten' (S34) and 'Kitasaisho' (S31)—showed the same sequence which was identical to a type of sequence from WPR (AB920144, Table 3). The PR produced in Nara prefecture which is usually called 'Yamato Shakuyaku' is believed to be derived from 'Bonten'; however, two samples (D9, D42) out of the five produced in Nara prefecture also belonged to the RPR subgroup, but not the WPR subgroup. Meanwhile, analysis of five individual samples which were randomly picked from a crude drug sample produced in Niigata prefecture (D6-1–5) achieved five types of ITS sequences; however, none were the same as the Japanese medicinal cultivars. Such sequences were detected in diverse horticultural cultivars of *P. lactiflora* (unpublished data), which suggested that PR from Japanese markets included not only roots of the medicinal cultivars but also roots of the horticultural cultivars of *P. lactiflora*.

Chemical comparison on the basis of quantitation of 8 main components

The contents of 8 main components were quantitatively analyzed to clarify the chemical properties of *P. lactiflora*, *P. veitchii*, *P. anomala*, and the crude drug samples including WPR, PR and RPR. Within the commercial samples derived from *P. lactiflora*, the RPR samples produced in the northern parts of China had an obviously higher content of paeoniflorin and paeonol, but a lower content of albiflorin than WPR/PR produced in the southern parts of China and most of the PR produced in Japan. Among the 11 commercial samples of WPR collected from various places in China, eight samples contained < 20 mg/g of paeoniflorin. In particular, the three samples (D18, D22, D24) had extremely low content of paeoniflorin (< 1.5 mg/g). When the HPLC chromatograms of these 3 samples were compared with others, a conspicuous peak with a retention time at approximately 10.4 min was

commonly observed in these 3 samples, which was not detected in the PR collected from the Japanese markets. Wang et al. [14] reported that an artifact compound, paeoniflorin sulphonate, could be detected in WPR which has been processed by sulfur fumigation (a traditional process for crude drugs in traditional Chinese medicine). Further analysis using LC/MS clearly indicated this notable peak was paeoniflorin sulphonate (data not shown). In the HPLC chromatograms of more than half of the WPR collected from Chinese markets (indicated in Fig. 3), the paeoniflorin sulphonate peak was detected. This result indicated that the WPR available from Chinese markets was usually processed by sulfur fumigating which resulted in an extremely low content of paeoniflorin; however, the PR available from Japanese markets did not undergo this process (Table 4).

Table 4 Contents of 8 compounds in *Paeonia* species and crude drug samples of WPR, PR and RPR

Code no.	含量 Content (mg/g)							
	Paeoniflorin	Albiflorin	PGG	Gallic acid	Catechin	Benzoic acid	Paeonol	Methylgallate
D1	23.39	4.07	2.57	0.89	0.26	0.32	ND	ND
D2	16.00	3.41	1.91	0.39	ND	2.20	0.02	ND
D3	28.29	3.43	3.60	1.14	0.24	0.60	ND	Trace
D17	20.21	5.33	3.31	0.98	0.30	0.34	ND	ND
D23	9.12	5.92	3.72	0.91	0.46	0.13	ND	ND
D4	37.58	10.36	10.51	1.60	0.40	1.39	ND	ND
D16	18.82	5.21	8.37	2.15	Trace	0.72	0.05	ND
D18	1.11	4.93	2.72	0.97	0.53	0.26	Trace	ND
D22	0.53	3.16	2.05	0.88	0.53	0.17	ND	ND
D29	18.48	4.96	3.52	0.53	0.57	0.20	ND	ND
D24	1.21	5.87	3.87	1.60	0.68	0.17	ND	ND
P9-1	24.81	4.77	5.71	0.84	0.29	0.68	ND	ND
P9-2	23.90	19.66	4.39	0.57	1.03	1.63	ND	ND
P9-3	20.02	1.47	5.11	1.39	0.23	0.20	ND	ND
D45	27.54	3.73	5.09	1.21	0.22	0.28	ND	ND
D46	34.42	2.77	7.38	0.95	Trace	1.20	0.03	ND
D47	43.46	13.05	9.60	1.66	0.48	1.90	ND	ND
D48	40.32	5.73	6.69	1.02	0.21	1.08	ND	ND
D53	29.24	3.29	7.29	1.38	Trace	2.03	ND	ND
D50	21.17	1.81	9.35	1.75	0.16	1.12	ND	ND
D51	22.10	3.78	8.87	1.88	ND	1.08	ND	ND
D52	21.82	5.28	7.44	1.57	ND	1.21	ND	ND
D10	29.97	2.67	5.83	1.00	0.33	1.28	ND	ND
D7	26.45	0.48	5.34	1.10	ND	1.47	ND	ND
D8	27.36	3.70	2.66	0.46	0.32	1.13	ND	ND
D6	33.42	3.80	4.10	0.81	0.43	1.12	Trace	ND
D9	41.99	4.11	3.75	0.77	1.24	0.76	ND	ND
D49	30.49	2.33	5.72	1.08	0.34	1.37	Trace	ND
P1	54.65	2.89	2.51	0.57	3.73	0.88	0.11	ND
D12	69.27	2.27	5.80	0.60	1.05	0.90	0.32	ND
D13	64.94	1.47	2.38	0.55	Trace	0.90	0.14	ND
D15	53.64	1.22	4.39	0.63	1.75	1.23	0.23	ND
D14	39.43	1.04	7.18	0.80	0.71	0.91	0.05	ND
D54	35.32	0.53	3.63	0.48	0.91	0.82	0.27	ND
P7	61.92	0.64	35.45	4.79	ND	1.01	ND	ND
D11	71.36	0.69	38.32	9.79	ND	0.82	ND	0.78
P4	8.42	2.65	11.08	2.25	ND	0.68	ND	ND
P5	13.00	0.24	11.29	1.62	ND	0.79	0.01	ND
P6	15.62	0.28	16.56	2.51	ND	0.81	ND	ND

ND, not detected; trace, lower than limit of quantitation

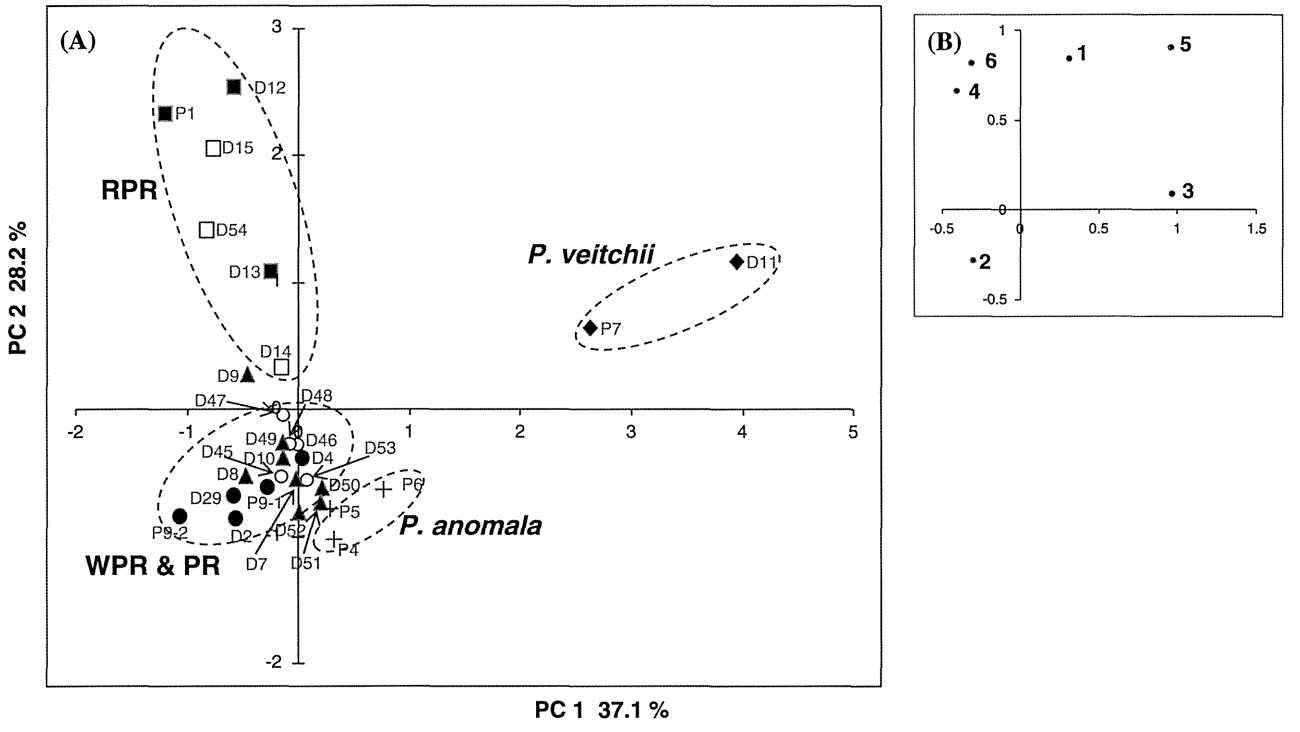

Fig. 4 Principal component analysis of chemical component data from *Paeonia* specimens and peony root samples. **a** Scores plot, *filled circle* WPR produced in China and P9-2, *open circle* PR produced in China, *filled triangle* PR produced in Japan, *filled square* RPR in Chinese market and P1, *open square* RPR in Japanese market. Above are *P. lactiflora*. *Plus symbol*, *P. anomala*; *filled diamond*, *P. veitchii*. **b** Loading plot; **1** paeoniflorin, **2** albiflorin, **3** PGG, **4** (+)-catechin, **5** gallic acid, **6** paeonol

The root of *P. veitchii* (P7), as well as the RPR sample derived from this species (D11) showed the highest content of paeoniflorin (6.19–7.14 %), PGG (3.55–3.83 %) and gallic acid (0.48–0.98 %), and clearly differed from the samples derived from *P. lactiflora*. In particular, the contents of PGG as well as gallic acid were twice as high as those in other species. Such characteristics in the chemical composition have been supported by the reported data [21]. In the roots of *P. anomala* (P4–P6), the content of paeoniflorin was very low and catechin was not detected.

Apart from the WPR samples which were thought to be processed by sulfur fumigation, the quantitative data of the 6 main compounds in *Paeonia* specimens and commercial samples were subjected to PCA analysis in order to compare the chemical characteristics of WPR and RPR derived from *P. lactiflora*, as well as the related species. The PCA scores plot is shown in Fig. 4a, where the first and second principal components accounted for 65.3 % of the total variance (PC1, 37.1 %; PC2, 28.2 %). The PCA loading plot (Fig. 4b) indicated that compounds **3** and **5** contributed much to the positive value of PC1, and compounds **1** and **4**–**6** contributed much to the positive value of PC2. The distribution of all the samples in the PCA scores plot (Fig. 4a) showed that four separated groups were clustered. Besides the respective groups of *P. veitchii* and *P. anomala*, samples derived from *P. lactiflora* were clearly classified into two groups—one group included RPR and the other group was composed of WPR, PR produced in China and most of the PR produced in Japan. By comparing the scores plot and loading plot, the characteristic chemical composition of the respective groups could be easily observed. The *P. veitchii* group which was different from other groups had a significantly high content of paeoniflorin, PGG and gallic acid. The RPR group had an obviously high content of paeonol, catechin and paeoniflorin, while the WPR/PR group had a relatively high content of albiflorin. Moreover, the grouping result in the PCA scores plot was in accordance with the clustering result based on the similarity of ITS sequences.

Conclusion

The present study determined the nucleotide sequence of the ITS region and conducted quantitative analysis of 8 main chemical components of four *Paeonia* species, as well as commercial samples of WPR, PR and RPR collected from Chinese and Japanese markets. The ITS sequence was informative and sufficiently powerful to discriminate *Paeonia* plants at either inter-species or intra-

species levels. Significant intra-species polymorphism of the ITS sequences was detected within *P. lactiflora*. Clustering analysis based on the ITS sequences of the samples showed that *P. lactiflora* formed a group which was completely separate from other *Paeonia* species. Within the *P. lactiflora* group, the WPR and PR produced in the southern parts of China and the RPR produced in the northern parts of China were clearly clustered into two subgroups. The nucleotides at positions 69, 458 and 523 upstream of the ITS sequence served as molecular markers to discriminate between WPR and RPR. The medicinal cultivars 'Bonten' and 'Kitasaisho', as well as half of the PR produced in Japan belonged to the WPR subgroup. Quantitative analysis of 8 main components clarified the chemical properties of three *Paeonia* species as well as the WPR and RPR derived from *P. lactiflora*. The root of *P. veitchii* and its derived RPR sample showed the highest content of paeoniflorin, PGG and gallic acid, which clearly differed from those derived from *P. lactiflora*. As for the samples derived from *P. lactiflora*, the RPR samples produced in the northern parts of China had an obviously higher content of paeoniflorin and paeonol, but a lower content of albiflorin than the WPR and PR produced in the southern parts of China and the PR produced in Japan. Chemical analysis indicated that the WPR available in Chinese markets was usually processed by sulfur fumigation, which resulted in an extremely low content of paeoniflorin. The present study indicated that WPR and RPR were not only geographically isolated, but also genetically and chemically separated. The ITS sequence provided a genetic index for their identification.

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